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We recently identified, cloned, and characterized a novel human tissue inhibitor of metalloproteinases-4, TIMP-4. To determine if TIMP-4 can modulate the *in vivo* growth of human breast cancers, we transfected a full-length TIMP-4 cDNA into MDA-MB-435 human breast cancer cells and studied the orthotopic growth of TIMP-4-transfected (TIMP4-435) versus control (neo-435) clones in the mammary fat pad of athymic nude mice. Overexpression of TIMP-4 inhibited the invasion potential of the cells in the *in vitro* invasion assay. When injected orthotopically into nude mice, TIMP-4 transfectants were significantly inhibited in tumor growth by 4-10-fold in primary tumor volumes; and in an axillary lymph node and lung metastasis as compared with controls. Purified recombinant TIMP4 protein demonstrated a strong inhibitory effect on the invasion of human breast cancer cells across reconstituted basement membranes. These results suggest the therapeutic potential of TIMP-4 in treating cancer malignant progression.

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I. BACKGROUND AND SIGNIFICANCE

Matrix metalloproteinases (MMPs) and their inhibitors tissue inhibitor of metalloproteinases (TIMPs) play a critical role in ECM homeostasis. Controlled remodeling of the ECM is an essential aspect during normal development, and deregulated remodeling has been indicated to have a role in the etiology of diseases such as arthritis, periodontal disease, and cancer metastasis (1-5). TIMPs are secreted multifunctional proteins that have anti-MMP activity as well as erythroid-potentiating and cell growth promoting activities. The stimulating effect on cell growth was initially recognized when TIMP-1 and TIMP-2 were identified having erythroid-potentiating activities (6-7). It is now clear through several recent reports that TIMP-1 and TIMP-2 are mitogenic for non-erythroid cells, including normal keratinocytes (8), fibroblasts (9), lung adenocarcinoma cells (10), and melanoma cells (10). The involvement of TIMPs in the activation of pro-MMP has also been demonstrated (11). In addition, the recent evidence indicates that TIMP family may be involved in steroidogenesis of rat testis and ovary indicating the potential role of TIMP in the reproduction (12). Four mammalian TIMPs have been identified so far: TIMP-1 (13), TIMP-2 (14), TIMP-3 (15-18), and the recently cloned TIMP-4 (19-23). The proteins are classified based on structural similarity to each other, as well as their ability to inhibit metalloproteinases.

The overproduction and unrestrained activity of MMPs have been linked to malignant conversion of tumor cells (24-27). The down-regulation of MMPs may occur at the levels of transcriptional regulation of the genes; activation of secreted proenzymes; and through interaction with specific inhibitor proteins, such as TIMPs. Their most widely recognized action is as inhibitors of matrix MMPs. Thus, the net MMP activity in the ECM is the result of the balance between activated enzyme levels and TIMP levels. Augmented MMP activity is associated with the metastatic phenotype of carcinomas, especially breast cancer (28-30). Decreased production of TIMP could also result in greater effective enzyme activity and invasive potentials (31-33). These results suggest that the inhibitory activity of TIMPs might be important in inhibiting tumor malignant progression leading to invasion and metastasis, and therefore lead one to expect that an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. In fact, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMP expression or by an exogenous supply of TIMPs (34-36). Alternatively, down-regulation of TIMP-1 and TIMP-2 have been reported to contribute significantly to the tumorigenic and invasive potentials of the cells (31-33).

In addition to inhibiting tumor cell invasion and metastasis, several previous studies have also demonstrated that TIMPs, either expressed endogenously or added exogenously, inhibit primary tumor growth of several different cells including c-Ha-ras transfected rat embryo fibroblasts (35), human gastric cancer cell KKLS (36), and murine B16 melanoma cells (37). Interestingly, both invasiveness and tumorigenic potential of murine 3T3 cells are conferred when TIMP production is impaired by an antisense approach (31-32). We had recently cloned and characterized a human TIMP-4 (19-23). We have produced and purified rTIMP4p from baculovirus infected cells (21). rTIMP4p was shown to inhibit MMP activity and tumor cell invasion across reconstituted basement membrane (21) .Transfection of TIMP-4 into human breast cancer cells inhibited the invasion potential of the cells in the *in vitro* invasion assay (23). When

injected orthotopically into nude mice, TIMP-4 transfectants were significantly inhibited in their tumor growth and axillary lymph node and lung metastasis as compared with controls (23). These results suggest the therapeutic potential of TIMP-4 in treating cancer malignant progression. These results suggest an important role of TIMP-4 in inhibiting primary tumor growth and progression leading to invasion and metastasis.

II. WORK ACCOMPLISHED

Specific Aim 2: To study the relevance of the TIMP-4 transfection to the invasion and metastasis of breast cancer cells in nude mice (see attached **reprint 1** for Figs. 1-5 and Tables 1 and 2):

Transfection and selection of TIMP-4 positive clones. In order to select a suitable breast cancer cell line for TIMP-4 gene transfection, a panel of breast cancer cell lines was screened for analysis of TIMP-4 expression. As we previously demonstrated (19), Northern blot analysis failed to detect the TIMP-4 transcript in most breast cancer cell lines except MDA-MD-231 cells. The inability to pick up the TIMP-4 mRNA in most breast cancer cell lines by Northern blot may suggest that 1) the TIMP-4 gene may be only expressed very weakly in breast epithelial cells but is mainly expressed in stromal cells; or 2) the expression of the TIMP-4 gene may be down-regulated in breast cancers during the breast cancer malignant progression.

We selected MDA-MB-435 cell line as recipient for TIMP-4 gene transfection because: 1) it lacks detectable TIMP-4 transcript; and 2) it is relatively highly tumorigenic and metastatic in nude mice. The full-length TIMP-4 cDNA was inserted into pCI-neo mammalian expression vector. The resulting vector was transfected into MDA-MB-435 cells. The same cells were also transfected with the vector containing no insert as a control. MDA-MB-435 subclones transfected with TIMP-4 cDNA were designated TIMP4-435, and MDA-MB-435 subclones transfected with pCI-neo were designated neo-435. Clones were initially screened by *in situ* hybridization on slides with a specific TIMP-4 antisense probe (data not shown), and the positive clones were subjected to Northern blot analysis. Fifteen TIMP4-MDA-435 clones were picked up by *in situ* hybridization. Fig 1. shows the Northern blot analysis of TIMP-4 expression in selected clones. All four selected TIMP4-435 clones expressed TIMP-4 mRNA transcripts. In contrast, none of the parental cells or neo-435-15 clone produced any detectable TIMP-4 transcripts. No changes in morphology were observed in these clones.

Expression of MMP inhibitory activity. The anti-MMP activity of TIMP-4 transfected clones was characterized. Conditioned media (CM) from clones of TIMP4-435-12, TIMP4-435-20, neo-435-15 , and parental MDA-MB-435 cells were collected, concentrated, and analyzed for metalloproteinase inhibitory activity by soluble gelatin degradation assay (Fig. 2). About 64% and 54% inhibition of MMP2 gelationlytic activity were observed in CMs from TIMP4-435-20 and TIMP4-435-12 clones, respectively. Whereas, CM from parental MDA-MB-435 cells only possessed 10% inhibition of MMP2 activity. A slight increase of MMP2 activity was observed in CM from neo-435-15 cells. In addition, we have previously shown that the CMs from TIMP-4-producing clones contained a prominent MMP inhibitory activity at 22 kDa in a non-reducing SDS gel containing gelatin; in contrast, no such activity was observed in the CM from neo-435 cells (19). These data indicate: 1) the TIMP-4 positive clones secret a functional TIMP-4-

mediated anti-MMP activity; 2) almost no endogenous TIMP activities were detectable in neo-435 clones in the same conditions for detection of recombinant TIMP-4 activity.

In vitro growth of TIMP4-435 cells. To determine whether TIMP-4 expression affects the growth of MDA-MB-435 cells, the growth rates of TIMP4-435-12 and TIMP4-435-20 cells were compared to that of parental MDA-MB-435 cells and neo-435-15 cells in the monolayer culture. No significant differences in growth rate were observed among TIMP-4 positive and TIMP-4 negative cells (data not shown). TIMP4-435-20 cells showed slightly faster growth kinetics compared to TIMP-4 negative cells. At day 7, accumulating cell population was increased approximately 10% in TIMP4-435-20 cells compared to the parental MDA-MB-435 cells and neo-435-15 cells (data not shown). However, the differences were not significant.

Invasion potential of TIMP4-MDA-435 cells. We used an *in vitro* reconstituted basement membrane invasion assay to determine if TIMP-4 expression affects breast cancer cell invasion. Both parental MDA-MB-435 cells and neo-435-12 and neo-435-15 cells were moderately invasive. At the end of 24-h incubation, about 10% of MDA-MB-435 cells, 9.3% of neo-435-15 cells, and 6.9% of neo-435-12 cells had crossed the Matrigel barrier. A significant reduction in invasive potential was noted in two TIMP-4 expressing clones with percentages of invasion for TIMP4-435-12 and TIMP4-435-20 being 2.3% and 1.8%, respectively. To facilitate the comparison of the relative invasiveness between controls and TIMP-4 transfected clones in this study, all values were normalized to the percent invasion of parental MDA-MB-435 cells which was taken as 100% (Fig. 3A). In order to rule out the possibility that the different invasion potentials between TIMP-4 positive and TIMP-4 negative clones are due to the endogenous differences of individual clones, we treated parental MDA-MB-435 cells with either the pooled CM from two TIMP4-435 clones or the CM from neo-435 clones. As shown in Fig. 3B, when the cells were incubated with the medium containing 50% of the pooled CM from TIMP4-435 clones, cell invasion was inhibited 49%. A significant 88% inhibition of invasion was observed when the cells were treated with 100% of the pooled CM from TIMP4-435 clones. In contrast, the pooled CM from neo-435 clones has no significant effect on cell invasion at the same condition.

Effect of TIMP-4 transfection on tumorigenicity. To study the effect of TIMP-4 expression on tumorigenicity, we have picked three TIMP-4 positive clones: TIMP4-435-20, TIMP4-435-12, and TIMP4-435-4; and three TIMP-4 negative cells: parental MDA-MB-435, neo-435-15, and neo-435-12. Three independent experiments were done to confirm reproducibility, and the data from these experiments are summarized in Table 1. After a lag phase of 7-10 days, mice given implants of both TIMP-4 positive and TIMP-4 negative cells developed tumors. There was no difference in tumor incidence among the groups. Starting at about 25 days after inoculation, great level of tumor necrosis was observed in tumors derived from MDA-MB-435, neo-435-15, and neo-435-12 cells. The same breast cancer cells transfected with TIMP-4, however, were significantly inhibited in their tumor growth; and either no or low level of tumor necrosis was observed. The size of TIMP4-435-20 tumors was only 17% of that in parental MDA-MB-435 tumors, 37% of that in neo-435-15 tumors, and 23% of that in neo-435-12 tumors. The tumor growth of TIMP4-435-12 cells was also significantly reduced, with 32% and 42% inhibition of tumor size observed as compared to MDA-MB-435 and neo-435-15 tumors, respectively. Fig. 4 shows a representative experiment from parental MDA-MB-435, TIMP4-435-12, and TIMP4-435-20 tumors (experiment 1 in Table 1). After a lag phase of 15 days, tumors

from parental MDA-MB-435 cells increased in volume at an exponential rate. In contrast, the growths of TIMP4-435-20 and TIMP4-435-12 cells were dramatically inhibited.

One of the TIMP-4 positive clones, TIMP4-435-4, exerted the similar tumor growth in nude mice compared with TIMP-4 negative neo-435-12 clone (experiment 3 in Table 1). The loss of inhibitory effect correlates with the loss of TIMP-4 expression in this clone in the *in vivo* environment. Fig. 5 shows a Northern blot analysis for TIMP-4 transcripts from tumors developed in animals injected with TIMP4-435 clones, parental MDA-MB-435 cells, and neo-435 clone. The TIMP-4 transcript was found in TIMP4-435-20 and TIMP4-435-12 clones, whereas it was absent in parental MDA-MB-435 cells, TIMP4-435-4 cells, and neo-435-15 cells. The lack of TIMP-4 transcript in the TIMP4-435-4 cells indicates the loss of TIMP-4 expression in this clone in the *in vivo* condition.

Regional and metastatic tumor dissemination. To study tumor dissemination, H & E stained paraffin sections of axillary lymph nodes and lungs were examined for morphologic evidence of tumor cells by light microscopy. TIMP-4 positive TIMP4-435-20 and TIMP4-435-12 clones showed an average lower proportion of lymph node positivity of 27% and 32% as compared to the average of 72% from TIMP-4 negative MDA-MB-435, neo-435-15, and neo-435-12 cells (Table 1). TIMP-4 positive clones also yielded significantly less lung micro-metastasis than TIMP-4 negative clones with a combined 16% of lung positivity for TIMP4-435-20 and TIMP4-435-12 tumors as compared with a combined 40% for MDA-MB-435, neo-435-15, and neo-435-12 tumors.

Microvessel counts (MVCs) of primary tumors. The TIMP-4 negative tumors grew more rapidly and showed a higher rate of lymph node and lung metastasis than TIMP-4 positive tumors, despite a similar growth rate *in vitro* as compared to TIMP-4 positive cells. We performed MVC analysis in an attempt to investigate the discrepancy between these *in vivo* and *in vitro* results. Recent studies suggest that MVCs of paraffin sections of human breast cancers can be utilized as a measure of tumor angiogenesis, and that these MVC measurements are independent markers of the prognosis (38-39). The MVC method involves counting the number of immunohistochemical stained microvessel profiles per unit area in the region of tumor containing the most active angiogenesis and thus reflects vessel formation in "angiogenic hot spots." We compared MVC values, measured by both the highest individual MVC (peak MVC) and the average MVC of 4 fields with the highest vessel densities (average peak MVC), in TIMP-4 negative MDA-MB-435 and neo-435-15 tumors, and TIMP-4 positive TIMP4-435-20 and TIMP4-435-12 tumors. We found that the combined values of TIMP4-435-20 and TIMP4-435-12 tumors had only 49-54% peak and average peak MVCs of those for MDA-MB-435 and neo-435-15 tumors (Table 2). Thus, using MVC as the criterion, TIMP-4 inhibited the tumor angiogenesis.

Specific Aim 3: recombinant TIMP-4 protein (see attached **reprint 2** for Figs. 1-4 and Table 1).

Expression and purification of rTIMP4p. rTIMP4p was produced in Sf9 insect cells using the baculovirus expression system. A pVL94-based transfection vector PA2-GP/TIMP4 was constructed to generate the recombinant virus which was subsequently used to infect Sf9 cells. The optimal yield of rTIMP4p was obtained from the conditioned medium of the infected cells at 70 h post infection. The best purification of rTIMP4p was achieved by a 4-step chromatography including a strong cation chromatography, a weak cation chromatography, a

hydrophobic interaction chromatography, and a size exclusion column. The rTIMP4p eluted from a size exclusion column was stored in the buffer containing 50 mM sodium acetate and 100 mM NaCl, pH 5.8. When analyzed by SDS-PAGE, this preparation showed a single band at molecular weight of 23 kDa (Fig. 1A), which is consistent with the predicted molecular weight based on the calculation from the protein sequence (19-20). The purified 23 kDa protein was confirmed as TIMP-4 by Western blot using a specific anti-TIMP-4 antibody (Fig. 1B). The consistency of calculated molecular weight and the actual molecular weight of purified rTIMP4p suggests that there was no post-translational glycosylation for rTIMP4p. This is in agreement with the absence of glycosylation site for TIMP-4 (20). The specific activity of the recovery of rTIMP4p is summarized in Table 1. The yield of purified rTIMP4p was approximately 1.7 mg/2 x 10^7 cells.

Characterization of anti-MMP activities of rTIMP4p. The inhibitory activity of rTIMP4p on MMPs was analyzed by a soluble gelatin degradation assay. As shown in Fig. 2, when MMP-2 and MMP-9 were incubated with purified rTIMP4p at the mole ratio of 1 to 2, the gelatinlytic activities were inhibited 88% for MMP-2 and 66% for MMP-9, respectively. A similar pattern with higher magnitude of inhibition was also observed for TIMP-2, suggesting that TIMP-4 may be more specific for MMP-2 in a similar manner to TIMP-2.

Kinetic analysis of the inhibition of MMPs by rTIMP4p was performed in a continuous fluorometric assay with a quenched fluorescent peptide substrate. The inhibition kinetics of rTIMP4p were analyzed against human MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9. The MMPs were incubated with different concentrations of rTIMP4p. As shown in Fig. 3, the inhibitor concentrations that reached to 50% inhibition of MMP activities (IC_{50}) were determined to be 19, 3, 45, 8, and 83 nM for MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, respectively. Therefore, TIMP-4 is a potent inhibitor of all five tested MMPs and it has preference for MMP-2 and MMP-7.

Inhibition of invasion potential of human breast cancer cells. We have demonstrated that transfection of TIMP-4 cDNA into human breast cancer cells inhibited tumor cell invasion cross reconstituted basement membrane (Matrigel) (23). The effect of purified rTIMP4p on the invasion of MDA-MB-435 human breast cancer cells was investigated. MDA-MB-435 cells were moderately invasive. At the end of 24-h incubation, about 10% of MDA-MB-435 cells had crossed the Matrigel barrier. A significant reduction in invasive potential was noted when rTIMP4p was added at two different concentrations. The percentages of invaded cells were 1.5% for the cells treated with 10 nM of rTIMP4p and 0.6% for the cells treated with 100 nM of rTIMP4p, respectively. To facilitate the comparison of the relative invasiveness between controls and rTIMP4p-treated cells in this study, all values were normalized to the percent invasion of control MDA-MB-435 cells which were taken as 100% (Fig. 4).

In order to rule out the possibility that the different invasion potentials between the control cells and rTIMP4p-treated cells are due to the potential inhibitory effect of TIMP-4 on cell growth, we conducted growth rate experiments to determine whether addition of rTIMP4p affects the growth of MDA-MB-435 cells. When the cells were treated with 20 nM, 50 nM, and 100 nM of rTIMP4p in the DME medium containing 5% FCS (changing the fresh medium and rTIMP4p every two days) for seven days, no significant differences in growth rate were observed between the control and rTIMP4p-treated cells (data not shown). These results are consistent with the results on the similar growth rates of the control MDA-MB-435 cells and TIMP-4 transfected cells (23).

III. CONCLUSIONS

1. TIMP-4 transfected MDA-MB-435 human breast cancer cells released a functional anti-MMP activity.
2. Overexpression of TIMP-4 inhibited the invasion potentials of MDA-MB-435 cells in the *in vitro* invasion assay.
3. Expression of TIMP-4 did not affect the growth of MDA-MB-435 cells.
4. TIMP-4 expression significantly inhibited the primary tumor growth and lymph node and lung micro-metastasis of the cells in the orthotopic nude mice model.
5. Microvessel counts (angiogenesis) were significantly decreased in the primary tumors from TIMP-4 transfected cells as compared to TIMP-4 negative tumors, suggesting that TIMP-4 may inhibit the tumor angiogenesis.
6. We produced and purified rTIMP4p from baculovirus infected cells. The purified protein migrated as a single 23 kD band in SDS-PAGE.
7. We developed a specific anti-TIMP-4 peptide antibody. The affinity purified antibody immunochemically stained a 23 kD rTIMP4p band on Western blot.
8. The anti-MMP (matrix metalloproteinase) activity of rTIMP4p was confirmed by both reverse zymography and soluble substrate degradation assay. In addition, enzymatic kinetic studies were performed and revealed IC_{50} (concentration at 50% inhibition) of 3 nM, 47 nM, and 8 nM for MMP-2, MMP-9, and MMP-7, respectively.
9. Purified rTIMP4p demonstrated a strong inhibitory effect on the invasion of human breast cancer cells across reconstituted basement membranes.

Augmented MMP activities are associated with the metastatic phenotype of carcinomas, especially breast cancer (24-27). The clinical importance of MMPs during the tumor progression, emphasizes the need to effectively block MMPs and the subsequent tumor cell invasion. The inhibitory effect of TIMPs on MMP activity leads one to expect that an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. Indeed, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMP expression or by an exogenous supply of TIMPs (34-37). Alternatively, down-regulation of TIMP-1 and TIMP-2 have also been reported to contribute significantly to the tumorigenic and invasive potentials of the cells (32-33). These results suggest that an inhibitory activity of TIMPs play an important role in inhibiting tumor cell malignant progression leading to invasion and metastasis.

In the current studies, we have transfected TIMP-4 cDNA into human breast cancer cells. Overexpression of TIMP-4 resulted in decreased invasion potential in a Matrigel invasion assay and decreased growth and lymph node and lung metastasis in the mammary fat pads of nude mice. The decreased growth rate of TIMP-4 transfected tumors may be due, in part, to inhibition of tumor angiogenesis induced by TIMP-4. We also demonstrated an inhibitory effect of the purified rTIMP4p on the invasion of human breast cancer cells, which is consistent with the studies on the inhibition of cell invasion on the TIMP-4 transfected cells compared with the TIMP-4 negative control cells.

In the experimental Matrigel invasion assay, approximately 95% inhibition of invasion potential was achieved when the breast cancer cells were treated with 100 nM of rTIMP4p.

Similar inhibitory effects with much less magnitude were also reported for TIMP-1 (40) and TIMP-2 (41) on different tumor cells. The almost complete suppression of invasion potential of breast cancer cells by rTIMP4p suggests that the major matrix degradation proteinases required for the invasion of breast cancer cells in the Matrigel invasion assay are MMPs and their enzymatic activities can be effectively inhibited by TIMP-4. The inhibition of breast cancer cell invasion by both an exogenous supply of rTIMP4p and the endogenous expressed TIMP-4 suggest that the TIMP-4-mediated anti-invasion activity could be physiologically or pathologically relevant in the tumor micro-environment. Because it will be possible to design vectors that could be specifically targeted to tumors, the potential application of TIMP-4 as a cytostatic agent for the gene therapy treatment of cancer warrants further investigation.

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Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4

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We recently identified, cloned, and characterized a novel human tissue inhibitor of metalloproteinases-4, TIMP-4 (Greene *et al.*, 1996). To determine if TIMP-4 can modulate the *in vivo* growth of human breast cancers, we transfected a full-length TIMP-4 cDNA into MDA-MB-435 human breast cancer cells and studied the orthotopic growth of TIMP-4-transfected (TIMP4-435) versus control (neo-435) clones in the mammary fat pad of athymic nude mice. TIMP4-435 clones expressed TIMP-4 mRNA and produced anti-metalloproteinase (MMP) activity, while neo-435 clones did not express TIMP-4 mRNA or produce detectable anti-MMP activity. Overexpression of TIMP-4 inhibited the invasion potential of the cells in the *in vitro* invasion assay. When injected orthotopically into nude mice, TIMP-4 transfectants were significantly inhibited in tumor growth by 4–10-fold in primary tumor volumes; and in an axillary lymph node and lung metastasis as compared with controls. These results suggest the therapeutic potential of TIMP-4 in treating cancer malignant progression

Keywords: TIMP; MMP; mammary carcinoma; nude mice; angiogenesis

Introduction

The overproduction and unrestrained activity of MMPs have been linked to malignant conversion of tumor cells (Heppner *et al.*, 1996; Himelstein *et al.*, 1994; Bernhard *et al.*, 1994; Pyke *et al.*, 1993; Naylor *et al.*, 1994; Polette *et al.*, 1993; Urbanski *et al.*, 1992; Tryggvason *et al.*, 1993; Basset *et al.*, 1990, 1994; Hoyhtya *et al.*, 1994; Poulsom *et al.*, 1993). The down-regulation of MMPs may occur at the levels of transcriptional regulation of the genes; activation of secreted proenzymes; and through interaction with specific inhibitor proteins, such as TIMPs. TIMPs are secreted as multi-functional proteins that play pivotal roles in the regulation of extracellular matrix (ECM) metabolism. Their most widely recognized action is as inhibitors of matrix MMPs. Thus, the net MMP activity in the ECM is the result of the balance between activated enzyme levels and TIMP levels. Augmented MMP activity is associated with the metastatic phenotype of carcinomas, especially breast cancer (Tryggvason *et al.*, 1993; Basset *et al.*, 1990, 1994; Hoyhtya *et al.*, 1994; Poulsom *et al.*, 1993). Decreased production of TIMP could also result in greater effective enzyme activity and invasive potentials (Khokha *et al.*, 1989; Denhardt *et al.*, 1992; Mohanam *et al.*, 1995). These results suggest that the inhibitory activity of TIMPs might be important in inhibiting tumor malignant progression leading to invasion and metastasis, and therefore lead one to expect that an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. In fact, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMP expression or by an exogenous supply of TIMPs (Tsuchiya *et al.*, 1993; Imren *et al.*, 1996; Watanabe *et al.*, 1996; Khokha *et al.*, 1994; Khokha and Zimmer *et al.*, 1992; DeClerck *et al.*, 1992). Alternatively, down-regulation of TIMP-1 and TIMP-2 have been reported to contribute significantly to the tumorigenic and invasive potentials of the cells (Khokha *et al.*, 1989; Denhardt *et al.*, 1992; Mohanam *et al.*, 1995).

In addition to inhibiting tumor cell invasion and metastasis, several previous studies have also demonstrated that TIMPs, either expressed endogenously or added exogenously, inhibit primary tumor growth of several different cells including c-Ha-ras transfected rat embryo fibroblasts (Imren *et al.*, 1996), human gastric cancer cell KKLS (Watanabe *et al.*, 1996), and murine B16 melanoma cells (Khokha *et al.*, 1994). Interestingly, both invasiveness and tumorigenic potential of murine 3T3 cells are conferred when TIMP production is impaired by an antisense approach (Khokha *et al.*, 1989; Denhardt *et al.*, 1992). We have recently identified and cloned a novel human tissue inhibitor of metalloproteinases, TIMP-4 (Greene *et al.*, 1996). In this study, we have investigated the effect of TIMP-4 on the tumorigenic, invasive, and metastatic behaviors of human breast cancer cells. We demonstrated here for the first time that the *in vitro* invasive and *in vivo* tumorigenic and metastatic potentials of human breast cancer cells can also be inhibited by overexpression of a single TIMP.

Results

Transfection and selection ofTIMP-4 positive clones

In order to select a suitable breast cancer cell line for TIMP-4 gene transfection, a panel of breast cancer cell lines was screened for analysis of TIMP-4 expression. As we previously demonstrated (Greene *et al.*, 1996), Northern blot analysis failed to detect the TIMP-4

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transcript in most breast cancer cell lines except MDA-MD-231 cells. The inability to pick up the TIMP-4 mRNA in most breast cancer cell lines by Northern blot may suggest that: (1) the TIMP-4 gene may be only expressed very weakly in breast epithelial cells but is mainly expressed in stromal cells; or (2) the expression of the TIMP-4 gene may be down-regulated in breast cancers during the breast cancer malignant progression.

We selected MDA-MB-435 cell line as recipient for TIMP-4 gene transfection because: (1) it lacks detectable TIMP-4 transcript; and (2) it is relatively highly tumorigenic and metastatic in nude mice. The full-length TIMP-4 cDNA was inserted into pCI-neo mammalian expression vector. The resulting vector was transfected into MDA-MB-435 cells. The same cells were also transfected with the vector containing no insert as a control. MDA-MB-435 subclones transfected with TIMP-4 cDNA were designated TIMP4-435, and MDA-MB-435 subclones transfected with pCI-neo were designated neo-435. Clones were initially screened by *in situ* hybridization on slides with a specific TIMP-4 antisense probe (data not shown), and the positive clones were subjected to Northern blot analysis. Fifteen TIMP4-435 clones were picked up by *in situ* hybridization. Figure 1 shows the Northern blot analysis of TIMP-4 expression in selected clones. All four selected TIMP4-435 clones expressed TIMP-4 mRNA transcripts. In contrast, none of the parental cells or neo-435-15 clone produced any detectable TIMP-4 transcripts. No changes in morphology were observed in these clones.

Expression of MMP inhibitory activity

The anti-MMP activity of TIMP-4 transfected clones was characterized. Conditioned media (CM) from clones of TIMP4-435-12, TIMP4-435-20, neo-435-15 , and parental MDA-MB-435 cells were collected, concentrated, and analysed for metalloproteinase inhibitory activity by soluble gelatin degradation assay (Figure 2). About 64% and 54% inhibition of MMP2 gelationlytic activity were observed in CMs from TIMP4-435-20 and TIMP4-435-12 clones, respectively. Whereas, CM from parental MDA-MB-435 cells only possessed 10% inhibition of MMP2 activity. A slight increase of MMP2 activity was observed in CM from neo-435-15 cells. In addition, we have previously shown that the CMs from TIMP-4-producing clones contained a prominent MMP inhibitory activity at 22 kDa in a non-reducing SDS gel containing gelatin; in contrast, no such activity was observed in the CM from neo-435 cells (Greene *et al.*, 1996). These data indicate: (1) the TIMP-4 positive clones secret a functional TIMP-4-mediated anti-MMP activity; (2) almost no endogenous TIMP activities were detectable in neo-435 clones in the same conditions for detection of recombinant TIMP-4 activity.

In vitro *growth of TIMP4-435 cells*

To determine whether TIMP-4 expression affects the growth of MDA-MB-435 cells, the growth rates of TIMP4-435-12 and TIMP4-435-20 cells were compared to that of parental MDA-MB-435 cells and neo-435-15 cells in the monolayer culture. No significant differences in growth rate were observed among TIMP-4 positive and TIMP-4 negative cells (data not shown).

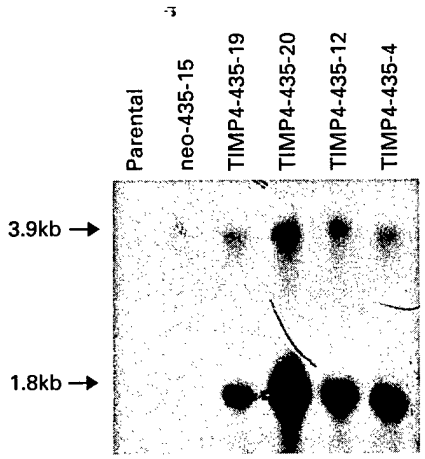

Figure 1 Northern blot analysis of TIMP-4 transfection of MDA-MB-435 human breast cancer cells. MDA-MB-435 cells were transfected with either pCITIMP4 plasmid containing full-length TIMP-4 cDNA or control pCI-neo plasmid, and the TIMP-4 positive clones were selected as described in Materials and methods. Total RNAs were isolated from parental MDA-MB-435 cells, one control pCI-neo transfected clone (clone 15), and four TIMP-4 transfected clones (clones 19, 20, 12, 4), and then subjected to Northern blot analysis with random primer-labeled full-length TIMP-4 probe. The integrity of the RNAs and loading control were ascertained by direct visualization of the 18 S rRNA bands in stained gel (data not shown)

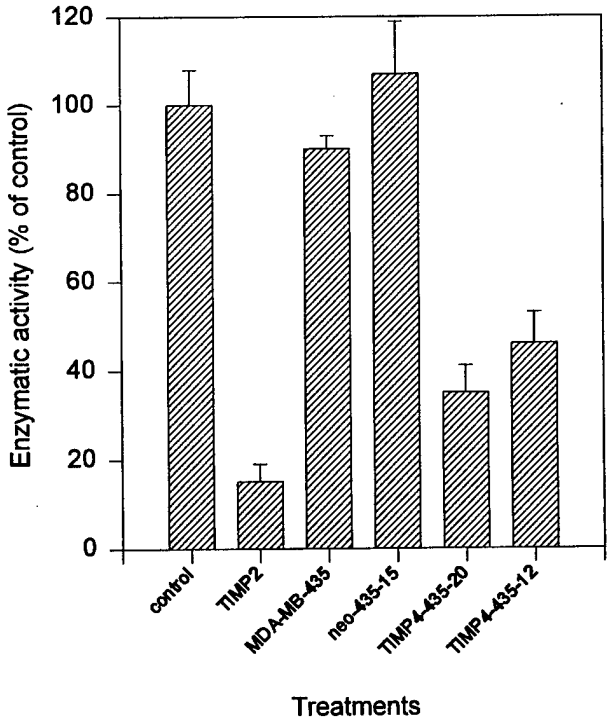

Figure 2 Inhibition of MMP activity by TIMP-4 transformed cells. CMs were prepared from parental MDA-MB-435 cells, clones of neo-MDA-15, TIMP4-435-20, and TIMP4-435-12, concentrated, normalized, and analysed by soluble degradation assay against MMP2 as described in Materials and methods. Recombinant TIMP-2, at the molar ratio of two to one relative to MMP2, was used as a positive control. The enzymatic activity of MMP2 in the absence of inhibitor or CMs was taken as 100% activity and regarded as control. All the other values were expressed as a percentage of the control. The numbers represent the means $\pm$ s.e.s. of three tests

TIMP4-435-20 cells showed slightly faster growth kinetics compared to TIMP-4 negative cells. At day 7, accumulating cell population was increased approximately 10% in TIMP4-435-20 cells compared to the parental MDA-MB-435 cells and neo-435-15 cells (data not shown). However, the differences were not significant.

Invasion potential of TIMP4-435 cells

We used an *in vitro* reconstituted basement membrane invasion assay to determine if TIMP-4 expression affects breast cancer cell invasion. Both parental MDA-MB-435 cells and neo-435-12 and neo-435-15 cells were moderately invasive. At the end of 24 h incubation, about 10% of MDA-MB-435 cells, 9.3% of neo-435-15 cells and 6.9% of neo-435-12 cells had crossed the Matrigel barrier. A significant reduction in invasive potential was noted in two TIMP-4 expressing clones with percentages of invasion for TIMP4-435-12 and TIMP4-435-20 being 2.3% and 1.8%, respectively. To facilitate the comparison of the relative invasiveness between controls and TIMP-4 transfected clones in this study, all values were normalized to the percent invasion of parental MDA-MB-435 cells which was taken as 100% (Figure 3a). In order to rule out the possibility that the different invasion potentials between TIMP-4 positive and TIMP-4 negative clones are due to the endogenous differences of individual clones, we treated parental MDA-MB-435 cells with either the pooled CM from two TIMP4-435 clones or the CM from neo-435 clones. As shown in Figure 3b, when the cells were incubated with the medium containing 50% of the pooled CM from TIMP4-435 clones, cell invasion was inhibited 49%. A significant 88% inhibition of invasion was observed when the cells were treated with 100% of the pooled CM from TIMP4-435 clones. In contrast, the pooled CM from neo-435 clones has no significant effect on cell invasion at the same condition.

Effect of TIMP-4 transfection on tumorigenicity

To study the effect of TIMP-4 expression on tumorigenicity, we have picked three TIMP-4 positive clones: TIMP4-435-20, TIMP4-435-12 and TIMP4-435-4; and three TIMP-4 negative cells: parental MDA-MB-435, neo-435-15, and neo-435-12. Three independent experiments were done to confirm reproducibility and the data from these experiments are summarized in Table 1. After a lag phase of 7–10 days, mice given implants of both TIMP-4 positive and TIMP-4 negative cells developed tumors. There was no difference in tumor incidence among the groups. Starting at about 25 days after inoculation, great level of tumor necrosis was observed in tumors derived from MDA-MB-435, neo-435-15, and neo-435-12 cells. The same breast cancer cells transfected with TIMP-4, however, were significantly inhibited in their tumor growth; and either no or low level of tumor necrosis was observed. The size of TIMP4-435-20 tumors was only 17% of that in parental MDA-MB-435 tumors, 37% of that in neo-435-15 tumors, and 23% of that in neo-435-12 tumors. The tumor growth of TIMP4-435-12 cells was also significantly reduced,

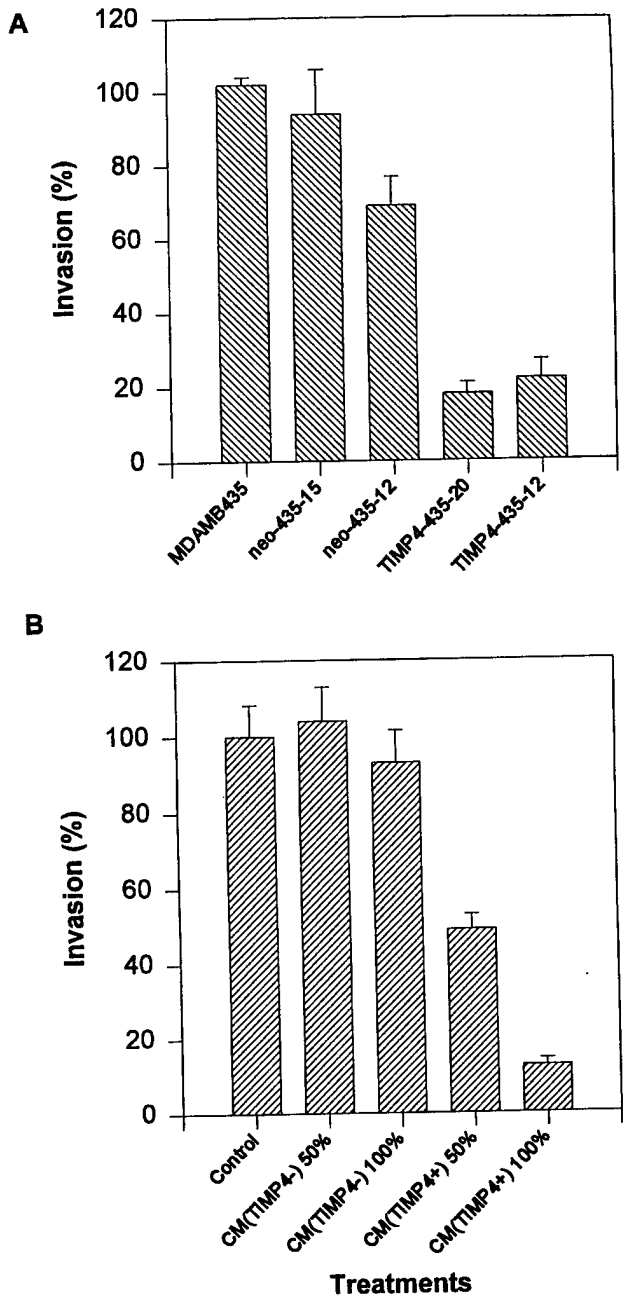

Figure 3 Inhibition of cell invasion by TIMP-4. (**a**) Comparison of invasion potentials of TIMP-4 positive and TIMP-4 negative cells. The invasion of MDA-MB-435 cells was used as control and was taken as 100%. The invasion potentials of all the other clones were expressed as a percentage of the control. (**b**) Inhibition of the invasion of MDA-MB-435 cells by CMs from TIMP-4 transfected clones. CM(TIMP4-) represents the equally pooled CMs from neo-435-12 and neo-435-20 cells. CM(TIMP4+) represents the equally pooled CMs from TIMP4-435-12 and TIMP4-435-20 cells. Control: cells were incubated in the regular DMEM-5% serum; CM (TIMP4−) 50%: cells were incubated in the medium containing 50% of CM(TIMP4−) plus 5% serum and 50% of regular DMEM-5% serum; CM (TIMP4−) 100%: cells were incubated in the medium containing 100% of CM(TIMP4−) plus 5% serum; CM(TIMP4+) 50%, cells were incubated in the medium containing 50% of CM(TIMP4+) plus 5% serum and 50% of the regular DMEM-5% serum; CM(TIMP4+) 100%: cells were incubated in the medium containing 100% of CM(TIMP4+) plus 5% serum. All values were normalized to the percent invasion of control cells which was taken as 100%. The numbers in both (**a**) and (**b**) represent the means ± s.e.s. of three cultures

Table 1 Effects of TIMP-4 expression on tumor sizes, lymph node status, and lung metastases of MDA-MB-435 cells

Experiment	*Treatment group*	*Tumor vol (mm^3) of primary size*	*Tumor incident tumor/total (%)*	*Lymph nodes Pos/total (%)*	*Lung metastases Pos/total (%)*
1	TIMP4-435-20	*260±84	14/16 (88)	3/14 (21)	0/7 (0)
	TIMP4-435-12	*495±99	15/16 (94)	4/15 (27)	2/8 (25)
	MDA-MB-435	1566±303	15/16 (94)	10/15 (67)	3/8 (38)
2	TIMP4-435-12	**860±365	10/12 (83)	4/10 (40)	2/5 (40)
	TIMP4-435-20	*770±230	12/12 (100)	4/12 (33)	1/6 (17)
	neo-435-15	2060±135	12/12 (100)	9/12 (75)	3/6 (50)
3	TIMP4-435-20	*580±223	11/12 (91)	3/11 (27)	0/6 (0)
	TIMP4-435-4	2200±819	12/12 (100)	6/12 (50)	2/6 (33)
	neo-435-12	2550±404	11/12 (91)	8/11 (73)	2/6 (33)

Four hundred thousand of the cells were injected at day one into the mammary fat pads, and tumor volumes and lymph node and lung micrometastasis were determined as described in Materials and methods. Volumes are expressed as means±s.e.s (number of tumors assayed). Experiment 1, total 16 injections for eight mice in each group, and the mice were sacrificed 32 days after injection. Statistical comparisons for TIMP-4 positive clones relative to TIMP-4 negative clones in the same experiment: *indicates $P<0.001$ for the mean tumor sizes; **indicates $P<0.01$ for the mean tumor sizes. Lymph node positivity of pooled TIMP4-435-20 tumors (experiments 1-3) *vs* pooled TIMP-4 negative MDA-435 (experiment 1), neo-435-15 (experiment 2), and neo-435-12 (experiment) tumors gave $P<0.029$; $P<0.032$ for pooled TIMP4-435-12 (experiments 1 & 2) *vs* combined TIMP-4 negative tumors. For lung metastasis, comparisons of pooled values of TIMP4-435-20 (experiments 1–3) and TIMP4-435-12 (experiments 1 & 2) *vs* MDA-MB-435, neo-435-15, and neo-435-12 gave $P<0.038$. Statistical comparison for primary tumors was analysed by Students *t* test. A chi-squared test was used for statistical analysis of lymh node and lung metastasis

with 32% and 42% inhibition of tumor size observed as compared to MDA-MB-435 and neo-435-15 tumors, respectively. Fig. 4 shows a representative experiment from parental MDA-MB-435, TIMP4-435-12, and TIMP4-435-20 tumors (experiment 1 in Table 1). After a lag phase of 15 days, tumors from parental MDA-MB-435 cells increased in volume at an exponential rate. In contrast, the growths of TIMP4-435-20 and TIMP4-435-12 cells were dramatically inhibited.

One of the TIMP-4 positive clones, TIMP4-435-4, exerted the similar tumor growth in nude mice compared with TIMP-4 negative neo-435-12 clone (experiment 3 in Table 1). The loss of inhibitory effect correlates with the loss of TIMP-4 expression in this clone in the *in vivo* environment. Figure 5 shows a Northern blot analysis for TIMP-4 transcripts from tumors developed in animals injected with TIMP4-435 clones, parental MDA-MB-435 cells, and neo-435 clone. The TIMP-4 transcript was found in TIMP4-435-20 and TIMP4-435-12 clones, whereas it was absent in parental MDA-MB-435 cells, TIMP4-435-4 cells, and neo-435-15 cells. The lack of TIMP-4 transcript in the TIMP4-435-4 cells indicates the loss of TIMP-4 expression in this clone in the *in vivo* condition.

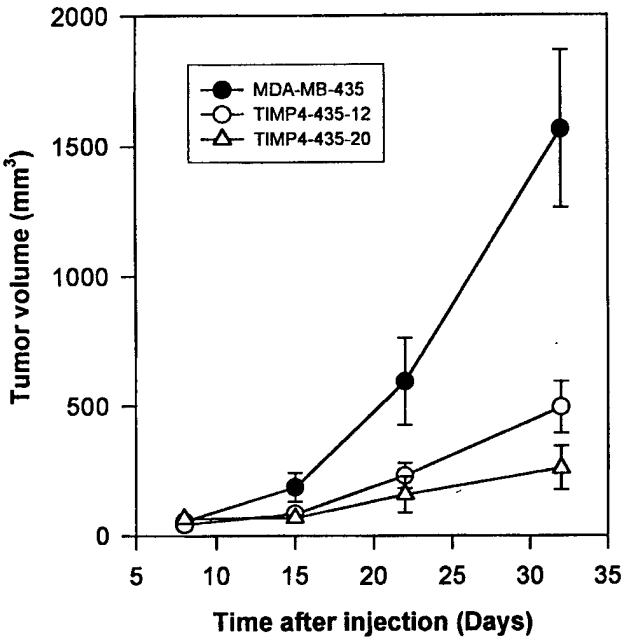

Figure 4 *In vivo* tumor growth of TIMP4-435-20, TIMP4-435-12, and parental MDA-MB-435 cells in the mammary fat pads of nude mice. Each point represents the mean±s.e. of tumors. Comparison of TIMP4-435-20 and TIMP4-435-12 tumors *vs* parental MDA-MB-435 tumors was significant ($P<0.001$). The tumor volume data from the last measurement were also presented in the experiment 1 of Table 1

Regional and metastatic tumor dissemination

To study tumor dissemination, H&E stained paraffin sections of axillary lymph nodes and lungs were examined for morphologic evidence of tumor cells by light microscopy. TIMP-4 positive TIMP4-435-20 and TIMP4-435-12 clones showed an average lower proportion of lymph node positivity of 27% and 32% as compared to the average of 72% from TIMP-4 negative MDA-MB-435, neo-435-15, and neo-435-12 cells (Table 1). TIMP-4 positive clones also yielded significantly less lung micro-metastasis than TIMP-4 negative clones with a combined 16% of lung positivity for TIMP4-435-20 and TIMP4-435-12 tumors as compared with a combined 40% for MDA-MB-435, neo-435-15, and neo-435-12 tumors.

Microvessel counts (MVCs) of primary tumors

The TIMP-4 negative tumors grew more rapidly and showed a higher rate of lymph node and lung metastasis than TIMP-4 positive tumors, despite a similar growth rate *in vitro* as compared to TIMP-4 positive cells. We performed MVC analysis in an attempt to investigate the discrepancy between these *in vivo* and *in vitro* results. Recent studies suggest that MVCs of paraffin sections of human breast cancers can be utilized as a measure of tumor angiogenesis, and that these MVC measurements are independent

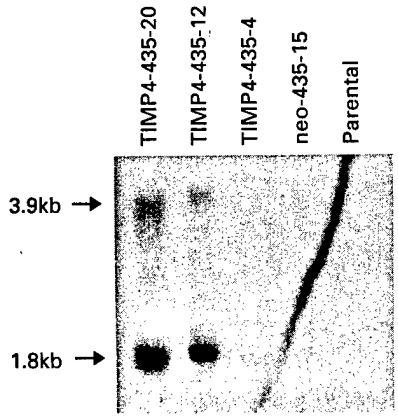

Figure 5 Analysis of TIMP-4 expression in tumor xenografts. RNAs were isolated from pooled tumor xenografts from parental MDA-MB-435 tumors, neo-435-15 tumors, TIMP4-435-20, TIMP4-435-12, and TIMP4-435-4 tumors, normalized, and analysed by Northern blot. The integrity and the loading control of the RNAs were ascertained by direct visualization of the 18 S rRNA bands in stained gel (data not shown)

markers of the prognosis (Weidner and Semple *et al.*, 1991; Weidner and Folkman *et al.*, 1992). The MVC method involves counting the number of immunohistochemical stained microvessel profiles per unit area in the region of tumor containing the most active angiogenesis and thus reflects vessel formation in 'angiogenic hot spots'. We compared MVC values, measured by both the highest individual MVC (peak MVC) and the average MVC of four fields with the highest vessel densities (average peak MVC), in TIMP-4 negative MDA-MB-435 and neo-435-15 tumors, and TIMP-4 positive TIMP4-435-20 and TIMP4-435-12 tumors. We found that the combined values of TIMP4-435-20 and TIMP4-435-12 tumors had only 49–54% peak and average peak MVCs of those for MDA-MB-435 and neo-435-15 tumors (Table 2). Thus, using MVC as the criterion, TIMP-4 inhibited the tumor angiogenesis.

Table 2 Microvessel counts (MVCs) for primary tumors

Clone	*Peak MVC*	*Average peak MVC*
MDA-MB-435	7.1±0.8 (15)	5.9±0.6 (15)
neo-435-15	6.5±0.7 (12)	5.4±0.7 (12)
TIMP4-435-20	3.1±0.4 (37)	2.9±0.4 (37)
TIMP4-435-12	3.6±0.3 (25)	3.3±0.2 (25)

Tumor sections were stained and examined for MVC as described in Materials and methods. Peak MVC is the number of microvessels per field in the region of most active angiogenesis. Average peak MVC is the mean MVC of the 4-fields with the highest microvessel counts. Values listed are means±s.e.s. The number of tumors analysed are shown in parenthesis. Comparisons of pooled TIMP-4 positive tumors (20+12) *vs* pooled TIMP-4 negative MDA-MB-435 and neo-435-15 tumors gave $P<0.01$ for both peak MVC and average peak MVC

Discussion

We have overexpressed TIMP-4 in the highly malignant MDA-MB-435 human breast cancer cells, and examined the subsequent phenotypical changes on *in vitro* growth rates, invasive potentials, *in vivo* tumorigenicity, and metastasis. We showed that transfection of TIMP-4 cDNA into MDA-MB-435 cells results in a decreased rate of orthotopic tumor growth in nude mice. Tumors derived from TIMP4-435 clones also had significantly lower rates of regional lymph nodes and lung metastasis. The invasive potential of MDA-MB-435 cells cross Matrigel was significantly inhibited by overexpression of TIMP-4.

Transfection of the MDA-MB-435 cells with a TIMP-4 cDNA leads to increased expression of the TIMP-4 transcript and anti-MMP activity when compared to parental cell line and control cells. The reduced *in vitro* invasiveness of TIMP4-435 clones compared to control cells suggests that the production of TIMP-4 altered the invasive potential of breast cancer cells in this experimental model system. These results are consistent with the previous reports on the inhibition of the invasion by TIMP-1 (Matsuzawa *et al.*, 1996) and TIMP-2 (Albini *et al.*, 1991). While our results support a role for MMPs and their inhibitors in breast cancer cell invasion, we are aware that the Matrigel *in vitro* invasion assay may not be an accurate predictor of breast cancer cell invasion as it occurs *in vivo*. Although up-regulation of TIMP-4 in the Matrigel invasion assay clearly inhibited the *in vitro* invasion of MDA-MB-435 cells, the invasion process was not completely abrogated. One possible explanation for continued invasion in the presence of high levels of TIMP-4 is that more than one kind of TIMP is required to completely block the MMP activity responsible for cell invasion. Alternatively, the invasion of MDA-MB-435 cells in the Matrigel invasion assay could be mediated by some matrix degrading proteinase other than MMP.

Interaction between stromal and epithelial components in a paracrine fashion dictates the development of normal mammary gland (Lund *et al.*, 1996; Adam *et al.*, 1994). In addition, the stromal component from tumor-surrounding micro-environments may also play a crucial role not only in changing the biological behavior of tumor cells but also in determining the malignant pattern of tumor cells (Paget, 1989; Berrettoni *et al.*, 1986; Hlatky *et al.*, 1994; Shi and Liu, 1995). Controlled remodeling of the mammary ECM is an essential aspect in the process of normal development, and deregulated remodeling has been indicated to have a role in the etiology of breast cancer metastasis (Basset and Wolf *et al.*, 1994; Spyratos *et al.*, 1989; Barsky *et al.*, 1983). Although TIMPs were initially suggested to be important agents to suppress or prevent tumor progression leading to invasion and metastasis, recent observations have demonstrated an inhibitory effect of TIMPs on tumor growth (Tsuchiya *et al.*, 1993; Imren *et al.*, 1996; Watanabe *et al.*, 1996; Khokha *et al.*, 1994). Within the same content, the tumorigenicity of MDA-MB-435 cells was inhibited by TIMP-4. This TIMP-4-mediated *in vivo* tumor growth inhibition is somewhat conflicting to the *in vitro* similar growth rates of TIMP4-positive clones compared to TIMP4-negative clones. The slower *in vivo* growth of TIMP4-435 tumors may be explained, in part, by TIMP-4-mediated inhibition of angiogenesis. In an effort to support this hypothesis, we have measured the microvessel densities in the highly angiogenic regions of both TIMP-4 positive TIMP4-435-20 and TIMP4-435-12 tumors and TIMP-4 negative MDA-MB-435 and neo-435-15 tumors. Sections of TIMP-4 positive

tumors had approximately 50% microvessel densities as compared with TIMP-4 negative tumors ($P<0.01$) Angiogenic regulatory factors have been found to modulate the growth of human breast cancers in several orthotopic xenograft models. We have recently demonstrated that transfection of MDA-MB-435 cells with an angiogenic factor Scatter Factor (SF) increased tumor growth and angiogenesis (Lamszus *et al.*, 1997). MCF-7 cells, which normally require estrogen supplementation for sustained *in vivo* tumor growth in nude mice, exhibited progressive estrogen-independent tumor growth when pro-angiogenic fibroblast growth factors (FGF-1 and FGF-4) were transfected into and over-expressed in the cells (McLeskey *et al.*, 1993; Kern *et al.*, 1994; Haran *et al.*, 1994). Our data indicate that despite the lack of growth inhibition of TIMP-4 on breast cancer cells, TIMP-4 significantly inhibits tumor growth and metastasis presumably due to its anti-angiogenic activity. In fact, both TIMP-1 (Johnson *et al.*, 1994) and TIMP-2 (Ohba *et al.*, 1995; Murphy *et al.*, 1993) have been demonstrated to have an anti-angiogenic activity, and such inhibition of angiogenesis is mediated by inhibition of both endothelial cell proliferation (Murphy *et al.*, 1993) and migration (Johnson *et al.*, 1994).

Although our results are consistent with the previous studies that an excessive TIMP expression could function to block the tumor growth and progression, and therefore lead one to expect that the expression of TIMPs should be down-regulated during the breast cancer malignant progression. However, both *in situ* hybridization and immunohistochemical staining have demonstrated TIMP-2 expression in the stroma surrounding breast carcinomas but not in benign breast tissues (Hoyhtya *et al.*, 1994; Poulsom *et al.*, 1993; Visscher *et al.*, 1994). In particular, it was demonstrated that the clinical outcome of breast cancer is more closely related to the presence of TIMP-2 in the peri-tumoral stroma than to the corresponding MMPs (Poulsom *et al.*, 1993). The increased expression of TIMP-2 is usually reciprocally related to the increased expression of MMP-2 or MMP-9 (Hoyhtya *et al.*, 1994; Poulsom *et al.*, 1993; Visscher *et al.*, 1994), suggesting that when the excess of MMP are produced during the degradation of extracellular matrix, the levels of local TIMPs expression are usually elevated. Although the elevated levels of TIMP-1 and TIMP-2 in the stroma adjunct to the invasive breast carcinomas may indicate one of the biological mechanisms to try to balance the local tissue degradation, it may represent one of the subsequent acute host responses to the remodeling stimuli but not as a causative factor. Alternatively, the high level expression of TIMP in the breast cancer may favor the proposed bi-functional molecule of both anti-proteinase activity and growth factor activity (Stetler-Stevenson *et al.*, 1992).

In conclusion, transfection with TIMP-4 cDNA into human breast cancer cells induces: (1) decreased invasion potential in a Matrigel invasion assay; and (2) decreased growth and lymph node and lung metastasis in the mammary fat pads of nude mice. The decreased growth rate of TIMP-4 transfected tumors may be due, in part, to inhibition of tumor angiogenesis induced by TIMP-4. Because it will be possible to design vectors that could be specifically targeted to tumors, the potential application of TIMP-4 as a cytostatic agent for the gene therapy treatment of cancer warrants further investigation.

Materials and methods

Tissue culture

MDA-MB-435 human breast cancer cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were subcultured weekly.

Expression of TIMP-4 in human breast cancer cells

Human TIMP-4 full-length sequence was subcloned into the pCI-neo mammalian Expression Vector (Promega) downstream of the human cytomegalovirus promoter to generate the pCITIMP4 expression vector. Forty micrograms of pCITIMP4 or the control pCI-neo plasmid were transfected into MDA-MB-435 human breast cancer cells by calcium phosphate-mediated method as we previously described (Greene *et al.*, 1996). Thirty G418-resistant individual clones were selected in the selection medium containing 800 μg/ml of G-418, subcloned and characterized by *in situ* hybridization and Northern blot analysis.

Northern blot analysis

Detection of TIMP-4 mRNA expression was analysed by Northern blot as we described previously (Greene *et al.*, 1996).

Preparation of conditioned media (CM)

All the clones were maintained in subconfluent monolayers with 5% fetal calf serum. The medium was discarded and the monolayers washed twice with phosphate-buffered saline (PBS). The monolayers were cultured in the absence of serum, in DMEM supplemented with transferrin (1 mg/L), fibronectin (1 mg/L), and trace elements (Biofluides, Rockville, MD). After 24 h, the serum-free medium was discarded, and the cells were replenished with the fresh serum-free medium. The CMs were collected 30 h later. Media were then centrifuged at 1200 *g* and supernatants were saved and concentrated approximately five-fold using an Amicon hollow fiber concentrator with a 10 000 molecular weight cut off at 4°C. The protein concentrations of CMs were determined and normalized.

Gelatin degradation assay

Inhibition of enzymatic activity by CMs from TIMP-4 transfected clones was assayed by measuring degradation of [^{3}H]gelatin in a manner conceptually similar to our previous report (Shi *et al.*, 1993) with some minor modifications. Briefly, tritiated type I collagen was diluted with non-radioactive type I collagen and then denatured at 55°C for 10 min. Recombinant MMP2 was activated by incubation with 1 mM APMA for 20 min at 37°C. The activated enzyme was pre-incubated with CMs from either TIMP-4 negative clones or TIMP-4 transfected clones for 30 min. The final concentration of MMP2 was 0.4 μg/ml. These enzyme/CM mixtures were then incubated with the gelatin solution for 2 h at 37°C. The reaction was stopped by addition of EDTA at a final concentration of 18 mM. Proteins were precipitated on ice with 1% of trichloroacetic acid and 0.014% of tannic acid. The radioactive soluble peptide fragments were detected in a liquid scintillation counter.

In vitro *assay for cell growth*

Exponentially growing cultures of MDA-MB-435 clones were detached with trypsin, and the trypsin was neutralized with DMEM-5% serum. Cells were counted, diluted, and seeded in triplicate at 3000 cells per well (24-well plate) in 1 ml DMEM-5% serum. Cell growth was measured by counting cell numbers.

In vitro *invasion assay*

Ten-μm polycarbonate membranes were coated with 4 mg/ml growth factor-reduced Matrigel in medium as described previously (Sheng *et al.*, 1994) with some modifications. The cells were seeded at a density of 50 000 cells/ml/well in DMEM containing 5% serum. For testing the effect of CM on cell invasion, cells were incubated with either 50:50 medium (50% CM plus 5% serum and 50% DMEM-5% serum) or a 100% CM plus 5% serum. After incubation in a humidified incubator with 5% CO_2 at 37°C for 24 h, the medium as well as the cells were removed from the bottom chambers and concentrated on 3 μm polycarbonate membranes using Minifold 1 apparatus (Schleicher and Schuell). A LeukoSata Staining kit from Fisher was used to fix and stain the cells which were counted using a Zeiss microscope.

Tumor growth in athymic nude mice

Nude mouse tumorigenic assay was performed as we previously described (Shi and Lippman *et al.*, 1993). Briefly, parental MDA-MB-435 cells, TIMP4-435 clones, and neo-435 clones were grown to 80–90% of confluence in 150 cm^2 dishes and were harvested by incubation with 5 mM EDTA in PBS. The EDTA was neutralized with medium containing serum. The cells were washed twice with serum-free medium, counted, and re-suspended in serum-free DMEM at a concentration of 2.7×10^6 cells/ml. Approximately 0.4×10^6 cells (0.15 ml) were injected into 5–6 week old female athymic nude mice (Frederick Cancer Research and Development Center, Frederick, MD). Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples.

The animals were ear tagged. Primary tumor growth was assessed by measuring the volume of each tumor at weekly intervals. Tumor size was determined at intervals by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point. Animals were sacrificed between 32–40 days after injection, when the largest tumors reached about 15 mm in diameter.

Assessment of regional lymph node and lung metastasis

The axillary lymph nodes and lungs of sacrificed animals were excised, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for microscopic examination for morphologic evidence of tumor metastasis. Sections were reviewed and scored by two pathologists (AF and MW).

Microvessel counts (MVCs) of primary tumors

Analysis of MVCs in the regions of the most angiogenic area (angiogenic 'hot spots') was performed as previously described (Weidner and Semple *et al.*, 1991; Weidner and Folkman *et al.*, 1992) with some modifications (Lamszus *et al.*, 1997). Briefly, paraffin sections were stained with an anti-laminin antibody (Gibco, Gaithersberg, MD) (Wolff *et al.*, 1993). Vessel profiles were determined by counting four different areas of tumor containing the highest microvessel density. Two values were analysed for each tumor: (a) the peak MVC (single largest number of microvessels per 400× field); and (b) the average peak MVC (mean MVC of the 4400× fields with the largest numbers of microvessels). MVCs were analysed in blinded fashion.

Statistical analysis

Values were expressed as means ± standard errors (s.e.s.). Comparisons were made using the two-tailed Student's *t*-test. Where appropriate, the chi-squared test was used to compare proportions.

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Preparation and Characterization of Recombinant Tissue Inhibitor of Metalloproteinase 4 (TIMP-4)*

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TIMP-4, a novel human tissue inhibitor of metalloproteinase, was identified and cloned (Greene, J., Wang, M., Raymond, L. A., Liu, Y. E., Rosen, C., and Shi, Y. E. (1996) *J. Biol. Chem.* 271, 30375–30380). In this report, the production and characterization of recombinant TIMP-4 (rTIMP4p) are described. rTIMP4p, expressed in baculovirus-infected insect cells, was purified to homogeneity by a combination of cation exchange, hydrophobic, and size-exclusion chromatographies. The purified protein migrated as a single 23-kDa band in SDS-polyacrylamide gel electrophoresis and in Western blot using a specific anti-TIMP-4 antibody. Inhibition of matrix metalloproteinase (MMP) activities by rTIMP4p was demonstrated in five MMPs. Enzymatic kinetic studies revealed IC_{50} values (concentration at 50% inhibition) of 19, 3, 45, 8, and 83 nM for MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, respectively. Purified rTIMP4p demonstrated a strong inhibitory effect on the invasion of human breast cancer cells across reconstituted basement membranes. Thus, TIMP-4 is a new enzymatic inhibitor in MMP-mediated extracellular matrix degradation and may have therapeutic potential in treating cancer malignant progression.

MMPs[1] and their inhibitors TIMPs play a critical role in ECM homeostasis. Controlled remodeling of the ECM is an essential aspect of normal development, and deregulated remodeling has been indicated to have a role in the etiology of diseases such as arthritis, periodontal disease, and cancer metastasis (1–5). Four mammalian TIMPs have been identified so far: TIMP-1 (6), TIMP-2 (7), TIMP-3 (8–11), and the recently cloned TIMP-4 (12, 41). The proteins are classified based on structural similarity to each other, as well as their ability to inhibit metalloproteinases.

TIMPs are secreted multifunctional proteins that have anti-MMP activity as well as erythroid-potentiating and cell growth-promoting activities. The stimulating effect on cell growth was initially recognized when TIMP-1 and TIMP-2 were identified as having erythroid-potentiating activities (14, 15). It is now clear through several recent reports that TIMP-1 and TIMP-2 are mitogenic for non-erythroid cells, including normal keratinocytes (16), fibroblasts (17), lung adenocarcinoma cells (18), and melanoma cells (18). The involvement of TIMPs in the activation of pro-MMP has also been demonstrated (19). In addition, the recent evidence indicates that the TIMP family may be involved in steroidogenesis of rat testis and ovary indicating the potential role of TIMP in the reproduction (20).

The most widely appreciated biological function of the TIMPs is their role in the inhibition of cell invasions *in vitro* (21–24) and tumorigenesis (25–29) and metastasis *in vivo* (25–31). Since the net MMP activity is the result of the balance between activated enzyme levels and TIMP levels, an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. The tumor-suppressing activity of TIMP on primary tumor growth may be in part due to its anti-angiogenic activity. In fact, both TIMP-1 (32) and TIMP-2 (33, 34) have been demonstrated to have an anti-angiogenic activity, and such inhibition of angiogenesis is mediated by inhibition of both endothelial cell proliferation (34) and migration (32). The underlying molecular mechanism for the tumor suppressing activities of TIMPs, nevertheless, is thought to depend on their anti-MMP activities.

We had recently cloned and characterized a human TIMP-4 (12). Transfection of TIMP-4 into human breast cancer cells inhibited the invasion potential of the cells in the *in vitro* invasion assay (13). When injected orthotopically into nude mice, TIMP-4 transfectants were significantly inhibited in their tumor growth and axillary lymph node and lung metastasis as compared with controls (13). These results suggest the therapeutic potential of TIMP-4 in treating cancer malignant progression. These results suggest an important role of TIMP-4 in inhibiting primary tumor growth and progression leading to invasion and metastasis. In the present study, we have produced and purified rTIMP4p from baculovirus infected cells. rTIMP4p was shown to inhibit MMP activity and tumor cell invasion across reconstituted basement membrane.

MATERIALS AND METHODS

Reagents—Restriction enzymes were obtained from Boehringer Mannhem. Chromatography supplies were purchased from PerSeptive. All the other reagents are listed as indicated below.

Preparation of rTIMP4p from Baculovirus—The coding sequence of TIMP-4 is amplified using a standard polymerase chain reaction ap-

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[1] The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ECM, extracellular matrix; PAGE, polyacrylamide gel electrophoresis.

proach with the primers corresponding to the 5′ and 3′ sequences of the cDNA (5′ primer: GCT AGT GGA TCC CTG CAG CTG CGC CCC GGC G; 3′ primer: CGG CTT CTA GAA GGG CTG AAC GAT GTC AAC). The amplified fragment was gel-purified and digested with *Bam*HI and *Xba*I. To construct the recombinant baculovirus expression vector, the purified TIMP-4 polymerase chain reaction fragment was ligated into pA2-GP vector, which was derived from pVL94 (35). The resulting pA2-GP/TIMP4 vector was transfected into HB101 cells, and positive clones were identified using polymerase chain reaction screening and restriction enzyme analysis. The DNA sequence was confirmed by automatic DNA sequencing of both strands. A recombinant virus was produced and purified. Sf9 insect cells were infected with recombinant baculovirus in EXCEL401 serum-free medium (JRH Scientific) supplemented with 1% pencillin/streptomycin (Life Technologies, Inc.) and 1% fetal bovine serum (Life Technologies, Inc.). A 5-L Bioreactor was harvested 70 h post-infection, and cell viability was estimated to be 80%. The bioreactor supernatant was clarified using a continuous flow centrifuge (18,000 × *g*). The harvesting and subsequent chromatography steps were carried out at 4–8 °C.

Purification of rTIMP4p—As we previously described (13, 36), an MMP-2-mediated gelatin degradation assay was used to monitor the anti-MMP activity of rTIMP4p during the purification. The clarified supernatant was directly loaded onto a strong cation-exchange column (POROS HS50 from PerSeptive Biosystems; column dimensions, 3 × 10 cm) at a linear flow rate of 800 cm/h. The column was previously equilibrated with 50 mM sodium acetate, 100 mM NaCl, pH 5.8, for 10 column volumes. The bound proteins were eluted using the step elution of 200 mM NaCl, 400 mM NaCl, 600 mM NaCl, 1 M NaCl, and 2 M NaCl (in the same equilibration buffer as before). The 600 mM eluted fractions were found containing anti-MMP-2 activity. The active fractions were pooled and diluted with 50 mM sodium acetate, pH 5.8, to a conductivity of six millisiemens.

A weak cation-exchange column (POROS CM20 from PerSeptive Biosystems; column dimensions, 2 × 7 cm) was equilibrated with 10 column volumes of 50 mM Tris-HCl, 100 mM NaCl, pH 7.5. The pooled active fractions from the strong cation-exchange column were loaded onto the column at a linear flow rate of 840 cm/h. The bound proteins were first washed with the equilibration buffer and then eluted by a gradient elution using the equilibration buffer and the elution buffer containing 50 mM Tris-HCl and 1 M NaCl, pH 7.5. The gradient elution was conducted from 0.1 M to 1 M NaCl within a 10-bed volume. The active fractions were pooled, and NaCl was added to raise the conductivity to 200 millisiemens.

A moderate hydrophobic interaction chromatography column (POROS PE50 from PerSeptive Biosystems; column dimensions, 2 × 10 cm) was equilibrated with 10 column volumes of buffer containing 50 mM sodium acetate and 4 M NaCl, pH 5.8. The pooled fractions from the weak cation-exchange column were loaded onto the column at the flow rate of 100 ml/45 min. The bound proteins were eluted using 50 mM sodium acetate and 100 mM NaCl, pH 5.8. The eluted active fractions from the column were pooled together.

The pooled fractions from the hydrophobic column were loaded onto a size-exclusion column (Superdex S-200 from Pharmacia Biotech Inc.; column dimensions, 2.5 × 90 cm) with a flow rate of 20 ml/30 min. The was previously equilibrated using 50 mM sodium acetate and 100 mM NaCl, pH 5.8. The sizing fractions were analyzed by SDS-PAGE and Coomassie Blue staining, and the relevant fractions corresponding to a size of 23 kDa were pooled and tested for purity and anti-MMP activity.

Preparation of Anti-TIMP-4 Antibody—A peptide sequence corresponding to amino acids 207–225 of human TIMP-4 (12) was synthesized by an ABI 431A peptide synthesizer. Peptide synthesis reagents were from Advanced Chemtech, Louisville, KY. The purified peptide was conjugated to keyhole limpet hemocyanin (Sigma) via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce). New Zealand rabbits were immunized with the carrier-hapten conjugate in Freund's complete adjuvant (Pierce) followed by Freund's incomplete adjuvant at the recommended intervals. Animals were anesthetized and exsanguinated, and antibodies were separated from serum by ion exchange. For final purification, a TIMP-4 peptide affinity column was made by conjugating 20 mg of TIMP-4 peptide to 5 ml of AminoLink resin (Pierce Chemical Co.) using sodium cyanoborohydride (Sigma).

Western Blot—Samples were boiled in SDS/β-mercaptoethanol sample buffer and electrophoresed in 12% acrylamide-PAGE gels. Gels were blotted onto polyvinylidene difluoride membrane in 25 mM Tris, 192 mM glycine buffer, pH 8.3, containing 20% (v/v) methanol. Blots were blocked in 5% bovine serum albumin (Sigma) for 1 h. Primary antibodies were diluted 1:2000 in TTBS (30 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20). After incubation with the primary antibodies overnight at 4 °C, the blots were washed 4 × 10 min in TTBS, then incubated for 1 h in goat anti-rabbit IgG-horseradish peroxidase (Sigma) diluted 1:6000 in TTBS. The blots were then washed 4 × 10 min in TTBS, and the bands were visualized by chemiluminescence.

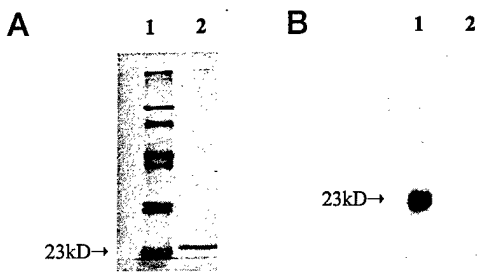

FIG. 1. **Purity and immnunoreactivity of the purified rTIMP4p.** *A*, SDS-polyacrylamide gel electrophoresis of purified rTIMP4p; *lane 1*, molecular mass markers; *lane 2*, rTIMP4p (50 ng). The homogeneity of the purified rTIMP4p was revealed by silver staining. *B*, immunoblot with a specific anti-TIMP-4 antibody. Experimental conditions are described under "Materials and Methods." *Lane 1*, 30 ng of purified rTIMP4p; *lane 2*, 30 ng of recombinant TIMP-2. No immunoreactivity toward TIMP-2 was observed.

Gelatin Degradation Assay—Inhibition of enzymatic activity by rTIMP4p was assayed by measuring degradation of [^{3}H]gelatin as we previously described (13).

Kinetic Studies—Active human neutrophil gelatinase B/92-kDa type IV collagenase (MMP-9) was purified as described (37). Human fibroblast collagenase (MMP-1), gelatinase A/72-kDa type IV collagenase (MMP-2), and stromelysin (MMP-3) were obtained from Dr. L. Jack Windsor of the University of Alabama at Birmingham (38). MMP-1 was autoactivated and converted to active catalytic domain (cdMMP-1) during the storage. Pro-MMP-2 (0.2 μM) was partially activated by three-times-repeated freeze and thaw cycles. Recombinant human active matrilysin (MMP-7) was kindly provided by Dr. H. E. Van Wart (37, 38). The substrate used for determining the TIMP-4 inhibition parameters was a quenched fluorescent substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) (39), which was purchased from Bachem. The final substrate concentration in the assays was 1 μM. The final enzyme concentrations in the assays were 5, 5, 4.9, 6.7, and 1.4 nM for MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, respectively. The enzymes were incubated with various concentrations of TIMP-4 at 25 °C for 30 min before adding the substrate to start the kinetic assay. The assays were carried out at 25 °C using a Perkin Elmer LS-5 fluorescence spectrometer (40).

In Vitro Invasion Assay—Inhibition of breast cancer cell invasions by purified rTIMP4p was evaluated in the Matrigel invasion assay with reconstituted basement membrane as we previously described (13).

RESULTS

Expression and Purification of rTIMP4p—rTIMP4p was produced in Sf9 insect cells using the baculovirus expression system. A pVL94-based transfection vector PA2-GP/TIMP4 was constructed to generate the recombinant virus which was subsequently used to infect Sf9 cells. The optimal yield of rTIMP4p was obtained from the conditioned medium of the infected cells at 70 h post-infection. The best purification of rTIMP4p was achieved by a 4-step chromatography including a strong cation chromatography, a weak cation chromatography, a hydrophobic interaction chromatography, and a size-exclusion column. The rTIMP4p eluted from a size exclusion column was stored in the buffer containing 50 mM sodium acetate and 100 mM NaCl, pH 5.8. When analyzed by SDS-PAGE, this preparation showed a single band at molecular mass of 23 kDa (Fig. 1*A*), which is consistent with the predicted molecular mass based on the calculation from the protein sequence (12, 41). The purified 23-kDa protein was confirmed as TIMP-4 by Western blot using a specific anti-TIMP-4 antibody (Fig. 1*B*). The consistency of calculated molecular mass and the actual molecular mass of purified rTIMP4p suggests that there was no post-translational glycosylation for rTIMP4p. This is in agreement with the absence of the glycosylation site for TIMP-4 (41). In addition, rTIMP4p gave a negative result in the glycosylation

TABLE I
Purification of rTIMP4p

Conditioned media (5 liters) from the recombinant baculovirus-infected Sf9 cells were collected and subjected to different chromatographies as described under "Materials and Methods." The activity of rTIMP4p was determined in the gelatin degradation assay. Protein present in the different steps of purification was estimated by Bio-Rad protein assay using bovine serum albumin as a standard.

Purification step	Total protein	Total activity[a]	Specific activity[b]	Percent recovery
	mg	*units*	*units/mg*	*%*
Media	305.0	19,678	64	100
HS-cation exchange	152.4	17,513	115	89
CM-cation exchange	69.1	14,759	214	75
PE-hydrophobic interaction	12.1	6,297	521	32
S-200 size exclusion	1.7	4,920	2,894	25

[a] One unit activity of rTIMP4p is defined as 60% inhibition of the enzymatic activity of recombinant MMP-2 (0.3 μg/ml) in the soluble gelatin degradation assay containing 20 μl of pooled active fraction.

[b] Specific activity was calculated by dividing total activity by total protein.

test using modified periodic acid-Schiff method (42) (data not shown). The specific activity of the recovery of rTIMP4p is summarized in Table I. The yield of purified rTIMP4p was approximately 1.7 mg/2 × 10^7 cells.

Characterization of Anti-MMP Activities of rTIMP4p—The inhibitory activity of rTIMP4p on MMPs was analyzed by a soluble gelatin degradation assay. As shown in Fig. 2, when MMP-2 and MMP-9 were incubated with purified rTIMP4p at the mole ratio of 1 to 2, the gelatinolytic activities were inhibited 88% for MMP-2 and 66% for MMP-9, respectively. A similar pattern with higher magnitude of inhibition was also observed for TIMP-2, suggesting that TIMP-4 may be more specific for MMP-2 in a manner similar to TIMP-2.

Kinetic analysis of the inhibition of MMPs by rTIMP4p was performed in a continuous fluorometric assay with a quenched fluorescent peptide substrate. The inhibition kinetics of rTIMP4p were analyzed against human MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9. The MMPs were incubated with different concentrations of rTIMP4p. As shown in Fig. 3, the inhibitor concentrations that reached to 50% inhibition of MMP activities (IC_{50}) were determined to be 19, 3, 45, 8, and 83 nM for MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, respectively. Therefore, TIMP-4 is a potent inhibitor of all five tested MMPs, and it has preference for MMP-2 and MMP-7.

Inhibition of Invasion Potential of Human Breast Cancer Cells—Previously, we demonstrated that transfection of TIMP-4 cDNA into human breast cancer cells inhibited tumor cell invasion cross-reconstituted basement membrane (Matrigel) (13). The effect of purified rTIMP4p on the invasion of MDA-MB-435 human breast cancer cells was investigated. MDA-MB-435 cells were moderately invasive. At the end of a 24-h incubation, about 10% of MDA-MB-435 cells had crossed the Matrigel barrier. A significant reduction in invasive potential was noted when rTIMP4p was added at two different concentrations. The percentages of invaded cells were 1.5% for the cells treated with 10 nM rTIMP4p and 0.6% for the cells treated with 100 nM rTIMP4p, respectively. To facilitate the comparison of the relative invasiveness between controls and rTIMP4p-treated cells in this study, all values were normalized to the percent invasion of control MDA-MB-435 cells which were taken as 100% (Fig. 4).

To rule out the possibility that the different invasion potentials between the control cells and rTIMP4p-treated cells are due to the potential inhibitory effect of TIMP-4 on cell growth, we conducted growth rate experiments to determine whether addition of rTIMP4p affects the growth of MDA-MB-435 cells. When the cells were treated with 0, 50, and 100 rTIMP4p in

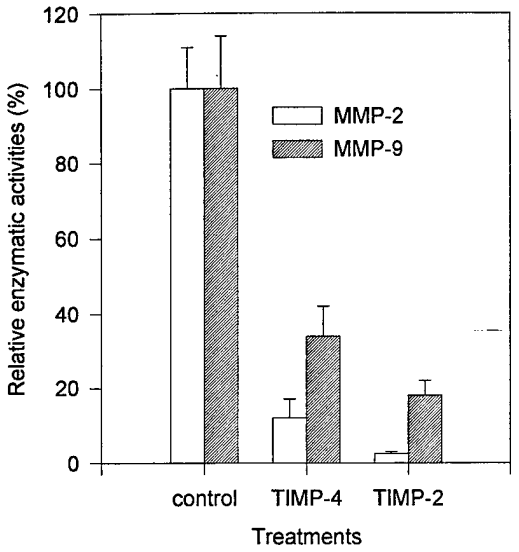

FIG. 2. **Inhibition of MMP-2 and MMP-9 by rTIMP4p in a soluble gelatin degradation assay.** MMP-2 (100 ng) and MMP-9 (120 ng) were incubated either with TIMP-4 or TIMP-2 at the mole ratio of 1 to 2 (MMP *versus* TIMP). The TIMP-mediated inhibition of enzymatic activity was expressed as percentage of the corresponding MMP activities without added inhibitors. The basal level enzymatic activity without the inhibitor was taken as 100%. The number represents the mean ± S.E. of three tests.

the Dulbecco's modified Eagle's medium containing 5% fetal calf serum (changing the fresh medium and rTIMP4p every 2 days) for 7 days, no significant differences in growth rate were observed between the control and rTIMP4p-treated cells (data not shown). These results are consistent with our previous report on the similar growth rates of the control MDA-MB-435 cells and TIMP-4-transfected cells (13).

DISCUSSION

Active recombinant TIMP-4 protein is required for characterization of its biochemical activity against MMPs and biological functions in inhibiting tumor growth and metastasis. Proteins of eukaryotic cells expressed in *Escherichia coli* are often generated as inactive, insoluble aggregates known as inclusion bodies and therefore require *in vitro* complicated refolding. In the present study, we expressed, purified, and characterized recombinant TIMP-4 protein prepared from baculovirus-infected insect Sf9 cells. The identity of rTIMP4p was confirmed by several criteria. First, as expected, the purified protein had a molecular mass of 23 kDa in SDS-PAGE, which is in close agreement with the calculated molecular mass of the 22.5-kDa protein, based on the mature protein of 195 amino acids after removal of the signal sequence (12). Second, the purified protein possessed a metalloproteinase inhibitory activity against MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9. Third, the purified protein can be recognized immunochemically by an affinity-purified specific anti-TIMP-4 polyclonal antibody. Fourth, the purified protein inhibited tumor cell invasion in the Matrigel invasion assay, and a similar effect was also reported for other TIMPs (21, 22).

We demonstrated here that human recombinant TIMP-4 can effectively inhibit human MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, with the IC_{50} values of 19, 3, 45, 8, and 83 nM, respectively. This relatively higher potency of TIMP-4 against MMP-2 than other MMP suggests that TIMP-4, like TIMP-2, is more specific for MMP-2. In fact, the predicted structure of the TIMP-4 shares 37% sequence identity with TIMP-1 and 51% identity with TIMP-2 (12). In addition, we also demonstrated a high affinity interaction between TIMP-4 and the C domain of MMP-2 and showed that TIMP-4 bound both full-length

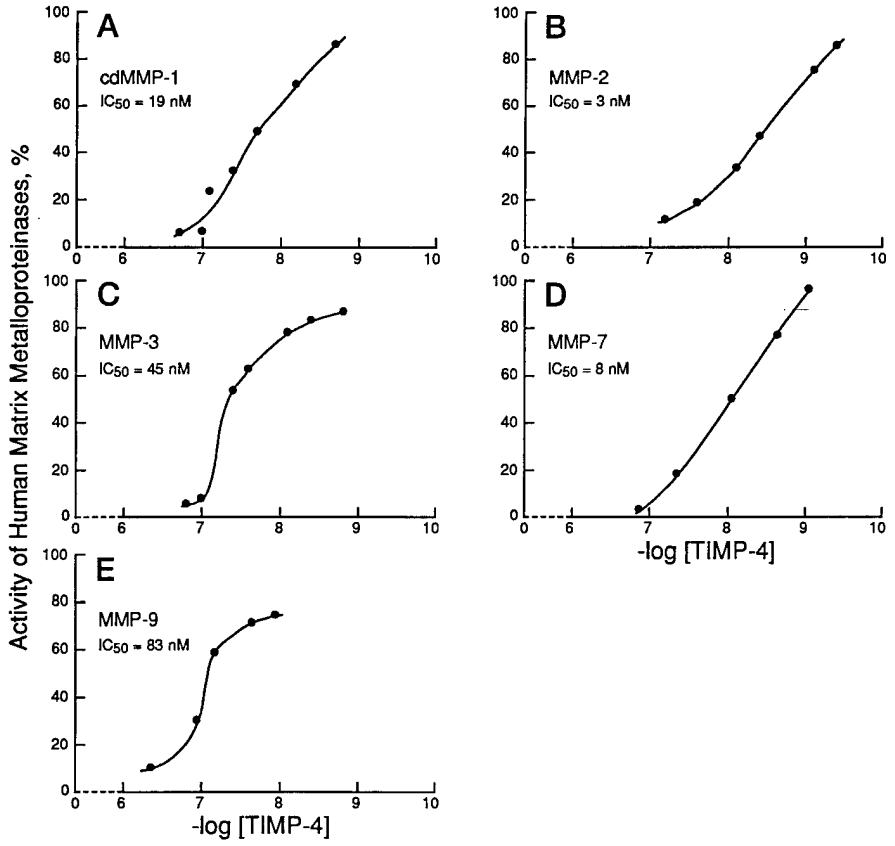

FIG. 3. **IC_{50} determination of TIMP-4 for MMP-1, MMP-2, MMP-3, MMP-7, and MMP-7.** Fixed concentrations of MMPs were incubated with different concentrations of rTIMP4p as described under "Materials and Methods." The concentrations of rTIMP4p varied for each individual MMP, ranging from the lowest 0.4 nM for MMP-2 to the highest 445 nM for MMP-9. The addition of increasing amounts of rTIMP4p to a constant amount of MMPs resulted in a decrease in the rate of the substrate hydrolysis.

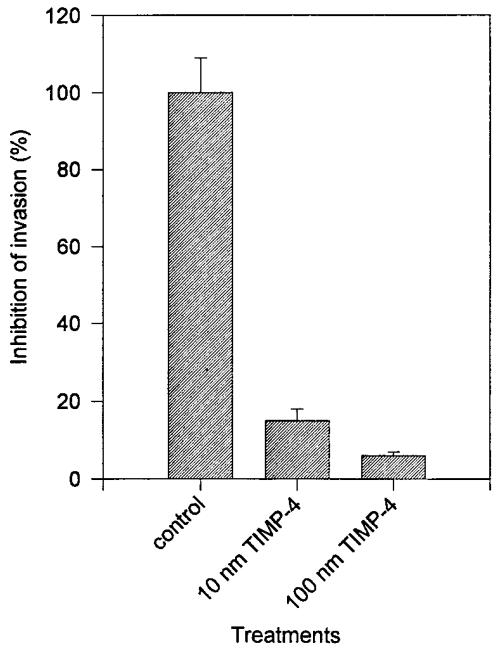

FIG. 4. **Inhibition of cell invasion by rTIMP4p.** The bottom wells of the invasion chamber were filled with Dulbecco's modified Eagle's medium containing 10% serum. MDA-MB-435 cells were seeded at a density of 50,000 cells/ml/well with or without rTIMP4p at the indicated final concentrations, and cell invasions were analyzed 24 h later as described previously (13). The invasion potential of the cells incubated without rTIMP4p was expressed as 100%. The number represents the mean ± S.E. of three cultures.

MMP-2 and the C domain of MMP-2 in a manner similar to TIMP-2 (43). Binding of MMP-2 to TIMP-4 was of high affinity with an apparent K_d of 1.7×10^{-7} M but sightly weaker than that to TIMP-2 (apparent K_d of 6.6×10^{-8} M) (43). These K_d differences are in agreement with the relatively more potent inhibitory effect of TIMP-2 on MMP-2 than that of TIMP-4 (Fig. 2). The overall sequence identity between TIMP-4 and other TIMPs suggests that TIMP-4 may inhibit MMPs through a similar mechanism by forming a strong noncovalent complex with a 1:1 stoichiometry (44). Although the inhibitory activity of TIMP is distributed throughout the molecule, the N-terminal regions of the TIMP family are highly conserved and thus may contribute to the inhibitory activities, and the C-terminal regions are divergent and may enhance the selectivity to the target enzymes (41, 45). A more detailed structural comparison indicated that TIMP-4 shares a relatively high identity with TIMP-2 particularly in the loops of 4 and 5 within the C-terminal domain (41). Thus, it is possible that TIMP-4 and TIMP-2 may share similar mechanistic and functional properties based on the sequence identity, similar enzymatic kinetics, and the high affinity binding to MMP-2 (43).

Augmented MMP activities are associated with the metastatic phenotype of carcinomas, especially breast cancer (46–49). The down-regulation of MMPs may occur at the levels of transcriptional regulation of the genes, activation of secreted proenzymes, and through interaction with TIMPs. The clinical importance of MMPs during the tumor progression emphasizes the need to effectively block MMPs and the subsequent tumor cell invasion. The inhibitory effect of TIMPs on MMP activity leads one to expect that an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. Indeed, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMP expression or by an exogenous supply of TIMPs (23, 24, 28–31). Alternatively, down-regulation of TIMP-1 and TIMP-2 have also been reported to contribute significantly to the tumorigenic and invasive potentials of the cells (25–27). These results suggest that an inhibitory activity of TIMPs play an important role in inhibiting tumor cell malignant progression leading to invasion and metastasis.

In this study, we demonstrated an inhibitory effect of the purified rTIMP4p on the invasion of human breast cancer cells, which is consistent with our previous studies on the inhibition of cell invasion on the TIMP-4-transfected cells compared with the TIMP-4 negative control cells (13). In the experimental Matrigel invasion assay, approximately 95% inhibition of invasion potential was achieved when the breast cancer cells were treated with 100 nM rTIMP4p. Similar inhibitory effects with much less magnitude were also reported for TIMP-1 (21) and TIMP-2 (22) on different tumor cells. The almost complete suppression of invasion potential of breast cancer cells by rTIMP4p suggests that the major matrix degradation proteinases required for the invasion of breast cancer cells in the Matrigel invasion assay are MMPs, and their enzymatic activities can be inhibited effectively by TIMP-4. The inhibition of breast cancer cell invasion by both an exogenous supply of rTIMP4p and the endogenous expressed TIMP-4 suggest that the TIMP-4-mediated anti-invasion activity could be physiologically or pathologically relevant in the tumor microenvironment.

Using *in situ* hybridization analysis, we have demonstrated a stromal expression of TIMP-4 mRNA in the fibroblasts surrounding the breast carcinomas.[2] The expression of TIMP-4 in the stroma adjunct to the breast carcinomas may indicate one of the host responses to try to balance the local tissue degradation due to the tumor cell invasion. Therefore, availability of the excess TIMP-4 relative to MMP (either by exogenous supply or endogenous expression) would create a microenvironment in the tumoral-stromal interface where the MMP-mediated ECM degradation and the subsequent tumor cell invasion can be inhibited by TIMP-4. While we are aware that the Matrigel *in vitro* invasion assay may not be an accurate predictor of breast cancer cell invasion as it occurs *in vivo*, we have recently demonstrated the TIMP-4-mediated anti-tumor and anti-metastasis activities of TIMP-4 transfected breast cancer cells in the animal model (13). These results support a role for MMPs and the inhibitor TIMP-4 in breast cancer cell invasion. Therefore, the potential therapeutic value of TIMP-4 for controlling cancer progression warrants further investigation.

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Specific, High Affinity Binding of Tissue Inhibitor of Metalloproteinases-4 (TIMP-4) to the COOH-terminal Hemopexin-like Domain of Human Gelatinase A

TIMP-4 BINDS PROGELATINASE A AND THE COOH-TERMINAL DOMAIN IN A SIMILAR MANNER TO TIMP-2*

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The binding properties of the newly described tissue inhibitor of metalloproteinases-4 (TIMP-4) to progelatinase A and to the COOH-terminal hemopexin-like domain (C domain) of the enzyme were examined. We present evidence for the first time of a specific, high affinity interaction between TIMP-4 and the C domain of human gelatinase A and show that TIMP-4 binds both progelatinase A and the C domain in a similar manner to that of TIMP-2. Saturable binding of recombinant C domain to TIMP-4 and to TIMP-2 but not to TIMP-1 was demonstrated using a microwell protein binding assay. The recombinant collagen binding domain of gelatinase A, comprised of the three fibronectin type II-like repeats, did not bind to TIMP-4, indicating that binding is mediated selectively by the C domain. Binding to TIMP-4 was of high affinity with an apparent K_d of 1.7×10^{-7} M but slightly weaker than that to TIMP-2 (apparent K_d of 0.66×10^{-7} M). Affinity chromatography confirmed the TIMP-4-C domain interaction and also showed that the complex could not be disrupted by 1 M NaCl or 10% dimethyl sulfoxide, thereby further demonstrating the tight binding. To verify the biological significance of this interaction, binding of full-length progelatinase A to TIMP-4 was investigated. TIMP-4 and TIMP-2 but not TIMP-1 bound specifically to purified TIMP-2-free human recombinant full-length progelatinase A and to full-length rat proenzyme from the conditioned culture medium of ROS 17/2.8 cells. Preincubation of the C domain with TIMP-2 was found to reduce subsequent binding to TIMP-4 in a concentration-dependent manner. Competition between TIMP-2 and TIMP-4 for a common or overlapping binding sites on the gelatinase A C domain may occur; alternatively TIMP-2 may prevent the binding of TIMP-4 by steric hindrance or induction of a conformational change in the C domain. We propose that the binding of progelatinase A to TIMP-4 represents a third TIMP-progelatinase interaction in addition to that of progelatinase A with TIMP-2 and progelatinase B with TIMP-1 described previously. This new phenomenon may be of important physiological significance in modulating the cell surface activation of progelatinase A.

Gelatinase A (72-kDa gelatinase, MMP-2, EC 3.4.24.24) belongs to the family of matrix metalloproteinases (MMPs),[1] a group of zinc-dependent enzymes, which together can degrade all components of the extracellular matrix (1, 2). Gelatinase A cleaves several connective tissue matrix proteins including denatured collagens (gelatins), native type IV, V, VII, X, and XI collagens, aggrecan, elastin, and fibronectin (3–8). The MMPs share common structural features (3, 9), and gelatinase A is typical of the family in that it is secreted as a proenzyme that is activated by proteolytic processing to remove an 80-amino acid propeptide (10, 11). The remainder of the NH_2-terminal domain (N domain) of gelatinase A contains a catalytic zinc-binding site that is common to all MMPs and also a fibronectin type II-like module triple repeat (12, 13) found only in the gelatinases that forms a collagen binding domain (CBD) (14–16). The hemopexin/vitronectin-like COOH-terminal domain (C domain) present in all MMPs except matrilysin does not appear to be required for catalysis by gelatinase A (17, 18), although it may have a role in binding substrates such as fibronectin and other extracellular matrix components such as heparin (19) and fetuin.[2]

Matrix metalloproteinase activity in the extracellular matrix is regulated by a family of specific inhibitors known as TIMPs (tissue inhibitor of metalloproteinases). Until recently, three members of this family had been characterized: TIMP-1 (20, 21), TIMP-2 (22, 23), and TIMP-3 (24, 25). The N domains of these proteins share structural similarities (26–28) and inhibit the activity of the MMPs by forming a 1:1 molar stoichiometric complex with the active enzyme, which is essentially irreversible (20, 29, 30). A new fourth member of the TIMP family (TIMP-4) has very recently been identified (31, 32); the predicted sequence of this protein shares a 37% homology with TIMP-1 but a 51% identity with TIMP-2 and TIMP-3 (31). Gelatinase A is unique among the MMPs in that the latent enzyme binds TIMP-2 to form a tightly bound 1:1 molar stoichiometric complex (22, 33–35), whereas only the active forms

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[1] The abbreviations used are: MMP, matrix metalloproteinase; N domain, NH_2-terminal domain; CBD, collagen-binding domain; C domain, COOH-terminal domain; TIMP, tissue inhibitor of metalloproteinases; MT, membrane type; rC domain, recombinant C domain; rCBD, recombinant collagen-binding domain; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

[2] B. Jarvis, A. King, J. Ochien, and C. M. Overall, manuscript in preparation.

of the other MMPs can bind this inhibitor. Binding of TIMP-2 to progelatinase A occurs via the C domains of the enzyme (17, 18, 34, 36) and inhibitor (37, 38); and with the other MMPs, an additional binding site for the N domain of TIMP-2 is also present in the catalytic domain of active gelatinase A (18, 22, 33, 34). A further binding site for the N domain of TIMP-2 is also found on the C domain of gelatinase A (39), although the biological significance of this interaction is not yet clear. The binding of TIMP-2 to the C domain of progelatinase A is involved in the activation mechanism of the enzyme. Unlike the other MMPs, progelatinase A fails to activate after treatment with proteinases such as plasmin, plasma kallikrein, neutrophil elastase, or cathepsin G (5, 8). Instead, a cell membrane-mediated activation process takes place (36, 40–42) in which the activator has been identified as a membrane type (MT) MMP (43, 44). Activated MT-MMP can act as a cell surface receptor for TIMP-2, which in turn acts as a receptor for progelatinase A, the latter binding via its C domain (43).

The interaction of progelatinase A with TIMP-2 appears to be specific in so far as a similar interaction does not take place between the latent enzyme and TIMP-1 as it does for gelatinase B (13, 45). However, the binding properties of TIMP-3 and TIMP-4 to progelatinase A have not yet been ascertained. We have therefore investigated the binding of gelatinase A to the newly identified TIMP-4 protein and identified the site of interaction utilizing recombinant domains of the human enzyme. These investigations have demonstrated specific binding of TIMP-4 to both full-length progelatinase A and to the C domain, which is closely similar to that of the TIMP-2-progelatinase A interaction.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Gelatinase A C Domain and CBD—Expression and purification of recombinant C domain (rC domain) and recombinant CBD (rCBD) was carried out essentially as described previously (14, 19). Briefly, the recombinant protein encoding the C domain and linker (Gly^{417}–Cys^{631}, exons 9–13) and the CBD (Val^{191}–Gln^{364}, exons 5–7) of human gelatinase A were both expressed with a short NH_2-terminal fusion tag that included an initiation methionine and a $(His)_6$ tag, using the expression vector pGYMX (14). Inclusion bodies from LE392 *Escherichia coli* expressing the recombinant proteins were solubilized in 6 M guanidine HCl, and recombinant protein was refolded in 0.1 M $Na_2B_4O_7$, pH 10, followed by dialysis into either 20 mM or 100 mM $Na_2HPO_4 \cdot 7H_20$, NaH_2PO_4, pH 8.0, 0.5 M NaCl. rC domain and rCBD were purified using Zn^{2+}-charged chelating Sepharose 6B (Pharmacia Biotech Inc.) affinity chromatography essentially as described previously (14, 19). rCBD eluted from the Zn^{2+}-chelate column was additionally applied to gelatin Sepharose 4B (Pharmacia) to select functionally folded CBD from the Zn^{2+}-chelating Sepharose elute (14). Protein yields were determined by the BCA assay (Pierce) and by spectroscopy after extinction coefficient determination (see below).

Expression and Purification of Recombinant TIMP-4—Expression and purification of recombinant TIMP-4 protein was carried out according to Liu *et al.*[3] Briefly, the DNA sequence encoding TIMP-4 was ligated into the pA2-GP vector derived from pVL94 (46), and the vector was transfected into HB101 cells to produce recombinant baculovirus, which was then used to infect Sf9 insect cells. TIMP-4 was purified from the clarified cell supernatant by cation exchange, hydrophobic, and size exclusion chromatography columns. A soluble gelatin degradation assay (47) was used to assay for anti-MMP activity of the recombinant TIMP-4 during purification. 200 μg of purified TIMP-4 was obtained for analysis.

SDS-PAGE—Heat denatured protein samples were separated under reducing (65 mM dithiothreitol) or nonreducing conditions by SDS-PAGE according to Laemmli (48) using 15% polyacrylamide gels. Protein bands were stained with Coomassie Brilliant Blue R-250. Samples analyzed by enzymography were electrophoresed nonreduced on 10% polyacrylamide gels copolymerized with 40 μg/ml gelatin (49).

[3] Y. E. Liu, M. Wang, J. Greene, J. Su, S. Ullrich, H. Li, S. Sheng, P. Alexander, Q. A. Sang, and Y. E. Shi, submitted for publication.

Mass Spectrometry and Amino Acid Analysis—The mass of the rC domain was measured by electrospray mass spectrometry using a PESCIEX API 300 after sample injection on a C18 high pressure liquid chromatography column at 50 μl/min. 10 μl of rC domain in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_20$, 1.5 mM KH_2PO_4, pH 7.4) was subjected to amino acid analysis (amino acid analyzer from Applied Biosystems) using norleucine as an internal standard. The extinction coefficient was then determined from measurement of the pmol content of the protein sample.

Microwell Protein Binding Assay—Binding of the rC domain to TIMP-4, TIMP-2, and TIMP-1 was determined using an enzyme-linked immunosorbent type assay (14) with bovine serum albumin as a negative control. Purified recombinant TIMP-2 and purified natural TIMP-1 were kindly supplied by Dr. Y. DeClerck (Children's Hospital, Los Angeles, CA) and Dr. I. Clark (Addenbrooke's Hospital, Cambridge, UK), respectively. Each protein at 0.5 μg/well in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% (w/v) NaN_3, pH 9.6, was coated on 96-well microtiter plates for 18 h at 4 °C. Wells were blocked with 2.5% (w/v) bovine serum albumin, 0.1% (w/v) NaN_3 in PBS for 1 h at 21 °C. Serially diluted rC domain (7.1×10^{-10} M to 1.15×10^{-5} M) was added in 100 μl of PBS for 1 h at 21 °C followed by washing with PBS, 0.05% (v/v) Tween 20 to remove unbound protein. Bound rC domain was quantitated using affinity purified anti-peptide αHis_6 antibody (19) followed by incubation with goat anti-rabbit alkaline phosphatase conjugated secondary antibody (H & L chains, Bio-Rad Laboratories) and *p*-nitrophenyl phosphate disodium (Sigma) as substrate. Absorbance measurements (405 nm) were made in a microplate reader (Thermomax, Molecular Devices). Specificity was confirmed by comparing binding of the rC domain with that of the rCBD because both proteins contain the same fusion tag and were expressed in the same *E. coli* strain.

Human recombinant full-length gelatinase A uncomplexed with TIMP-2 and predominantly in the latent form (M_r = 72,000) (kindly supplied by Dr. R. Fridman, Department of Pathology, Wayne State University, Detroit, MI) was applied at approximately 2 μg in 100 μl of PBS to microwell plates coated with TIMP-4, TIMP-2, or TIMP-1 with gelatin as a positive control and myoglobin as a negative control (0.5 μg/well). Gelatin was prepared from purified acid-soluble rat tail tendon type I collagen by heat denaturation at 56 °C for 30 min. Rat progelatinase A complexed with TIMP-2 in 100 μl of conditioned culture medium from ROS 17/2.8 cells (see below) was also applied to identically coated plates. Microwell plates were incubated at 21 °C for 2 h followed by collection of unbound material for enzymogram analysis. Any remaining unbound and nonspecifically bound enzyme was removed by washing plates with PBS, 0.05% (v/v) Tween 20. Bound gelatinase A was solubilized in 50 μl of 2 M urea, 2% (w/v) SDS, 0.125 M Tris-HCl, pH 6.8, and analyzed by gelatin enzymography. Unbound enzyme was also prepared and analyzed using the same buffer.

Affinity Chromatography—Binding and elution properties of the rC domain to TIMP-4 were also determined by affinity chromatography. A Zn^{2+}-charged chelating Sepharose 6B minicolumn (V_t = 75 μl) was equilibrated in PBS, and 50 μg of rC domain was applied. Saturation of the binding sites on the affinity matrix occurred resulting in a small amount of excess protein being recovered in the unbound material. 20 μg of purified TIMP-4 was applied, and elution was attempted with 1 M NaCl in 20 mM $Na_2HPO_4 \cdot 7H_20$, NaH_2PO_4, pH 7.4, 0.02% (w/v) NaN_3 followed by 10% (v/v) dimethyl sulfoxide in PBS and finally with 50 mM EDTA, pH 8.0. Chromatography fractions were analyzed by SDS-PAGE.

Cell Culture—ROS 17/2.8 cells were cultured in 75-cm^2 flasks (Becton Dickinson Labware) in α-minimum essential medium (Life Technologies, Inc.) supplemented with 10% (v/v) newborn calf serum (Life Technologies, Inc.). Confluent cells were washed twice in PBS, pH 7.4, and then cultured in serum-free medium with and without 20 μg/ml concanavalin A (Sigma). Conditioned culture medium was collected after an 18-h incubation to obtain active (41) and latent secreted gelatinase A complexed with TIMP-2.

RESULTS

Purification of Gelatinase A rC Domain—In agreement with our previous studies (19), purified rC domain electrophoresed as a single band on 15% SDS-PAGE gels with an apparent M_r of 26,500 under reducing condtions (Fig. 1*B*). Western blot analysis using two anti-peptide antibodies (19) verified the identity of the purified protein (not shown). The presence of an intact disulfide bond (Cys^{440} and Cys^{631}) was indicated by a downshift in apparent M_r of 0.8 under nonreducing conditions

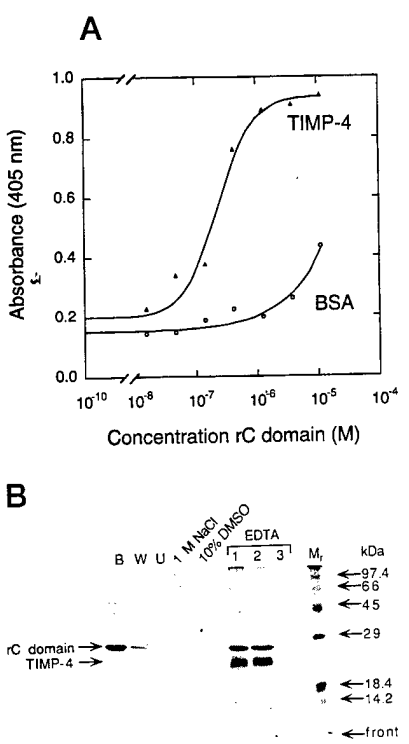

FIG. 1. **Binding of rC domain to TIMP-4.** *A*, TIMP-4 and bovine serum albumin (negative control) were coated on microtiter plates at 0.5 μg/well, and serially diluted rC domain (1.57×10^{-8} M to 1.15×10^{-5} M) was added in PBS, pH 7.4, followed by quantitation of bound rC domain as described under "Experimental Procedures." *B*, a Zn^{2+}-charged chelating Sepharose 6B minicolumn (V_t = 75 μl) was overloaded with 50 μg of rC domain (*lane B*, before chromatography) and then washed (*lane W*) with PBS. TIMP-4 (20 μg) was applied in PBS (*lane U*, unbound material), followed by sequential elution with 1 M NaCl (*1 M NaCl*), 10% (v/v) dimethyl sulfoxide (*DMSO*), and 50 mM EDTA, pH 8.0 (*EDTA fractions 1–3*), as shown. Aliquots from each fraction were electrophoresed on 15% SDS-PAGE gels and stained with Coomassie Brilliant Blue R-250. M_r, molecular mass markers in kDa as indicated; *front*, dye front.

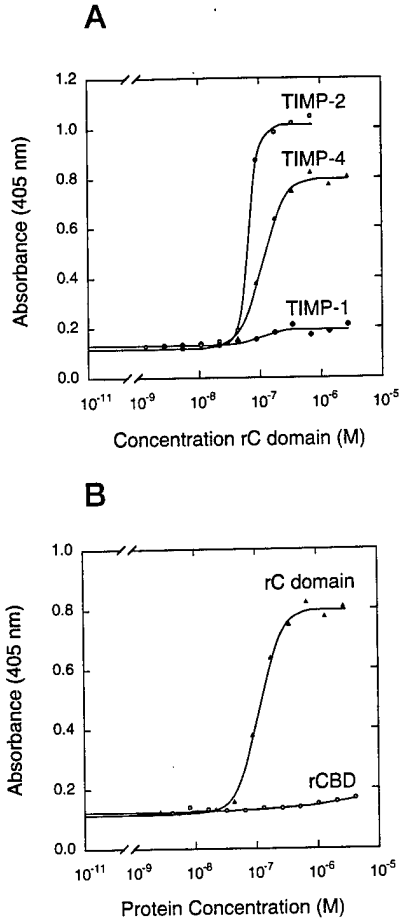

FIG. 2. **Specificity of binding of the rC domain to TIMP-4.** *A*, TIMP-4, TIMP-2, and TIMP-1 were coated on microtiter plates at 0.5 μg/well, and serially diluted rC domain (7.1×10^{-10} M to 2.83×10^{-6} M) was added in PBS, pH 7.4, followed by quantitation of bound rC domain as described under "Experimental Procedures." *B*, TIMP-4 was coated on microtiter plates at 0.5 μg/well, and serially diluted rC domain (2.74×10^{-9} M to 2.83×10^{-6} M) or gelatinase A rCBD (4.90×10^{-9} M to 4.99×10^{-6} M) was added in PBS, pH 7.4, followed by quantitation of bound protein as above.

(not shown). The absence of intermolecular disulfide-linked multimeric forms of the protein was shown by SDS-PAGE and Western blotting under nonreducing conditions. Electrospray mass spectrometry measured the precise mass of the protein to be 25,924 (within 1 Da of the predicted M_r of 25,924.9 for the NH_2-terminal methionine processed form), thereby confirming the fidelity of correct expression. The molar extinction coefficient determined from amino acid analysis of the purified protein was 5.10×10^4 M^{-1} cm^{-1}.

TIMP-4 Binding Properties of the rC Domain—Saturable binding of the rC domain to TIMP-4 in PBS was found with an apparent K_d of 1.7×10^{-7} M using the microwell protein binding assay; bovine serum albumin was used as a negative control (Fig. 1*A*). To confirm the TIMP-4-rC domain interaction, the binding of rC domain to TIMP-4 was investigated further by affinity chromatography. Fig. 1*B* shows that TIMP-4 was not detected in either the unbound fraction or in fractions eluted with 1 M NaCl or 10% dimethyl sulfoxide after application to a Zn^{2+}-chelating Sepharose minicolumn saturated with rC domain. TIMP-4 was recovered from the column by elution with 50 mM EDTA, pH 8.0, together with the bound rC domain (Fig. 1*B*).

Binding Specificity of rC Domain to TIMP-4—The binding of rC domain to TIMP-4 was compared with that to TIMP-1 and TIMP-2 using the microwell protein binding assay. Saturable binding to both TIMP-4 and TIMP-2 was observed (Fig. 2*A*) with the apparent K_d for the interaction with TIMP-2 being 0.66×10^{-7} M (c.f. 1.7×10^{-7} M for TIMP-4). These data indicate that both TIMP-4 and TIMP-2 bind the rC domain with similar high affinities but that the interaction with TIMP-2 was slightly stronger. No significant binding to TIMP-1 occurred (Fig. 2*A*), demonstrating that interaction of the rC domain with TIMP-4 and TIMP-2 was specific. Moreover, because the rCBD from gelatinase A did not bind TIMP-4 (Fig. 2*B*), this demonstrated that binding to TIMP-4 is mediated selectively by the C domain of gelatinase A.

TIMP-4 and TIMP-2 Bind at Shared or Overlapping Sites on the rC Domain—To investigate whether TIMP-4 and TIMP-2 bind at a common site on the gelatinase A C domain, 10 pmol of rC domain was incubated for 1 h at 21 °C in the presence of TIMP-2 (100, 40, 20, 10, 4, and 0 pmol). The reaction mix was then applied to microwell plates coated with TIMP-4 (0.5 μg, 20 pmol/well) for 1 h. Binding to TIMP-4 was reduced in a concentration-dependent manner by preincubation of the rC domain with increasing amounts of TIMP-2, and maximal inhibition was reached at a 2:1 molar ratio of TIMP-2 to rC domain (Fig. 3).

Binding of Full-length Progelatinase A to TIMP-4—Recombinant full-length progelatinase A uncomplexed with TIMP-2 was observed to bind TIMP-4 using the microwell protein binding assay (Fig. 4*A*), thereby confirming the biological relevance of the interaction found with the isolated rC domain. Binding to TIMP-2 and gelatin was also demonstrated (positive controls)

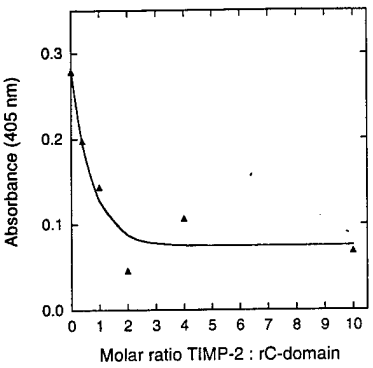

FIG. 3. **TIMP-4 and TIMP-2 compete for binding to the rC domain.** rC domain (1.00×10^{-7} M, 10 pmol) in PBS, pH 7.4, was incubated for 1 h at 21 °C with TIMP-2 (100, 40, 20, 10, 4, and 0 pmol) followed by application to microwell plates coated with TIMP-4 (0.5 μg, 20 pmol/well). Bound rC domain was quantitated as described under "Experimental Procedures."

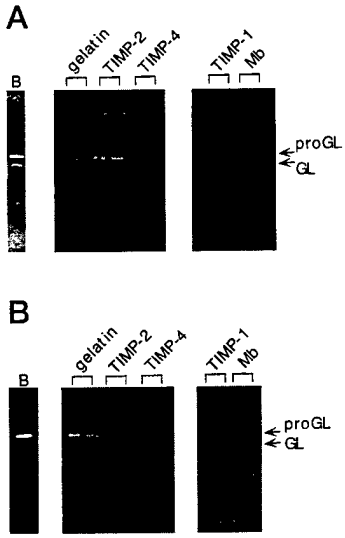

FIG. 4. **Binding of full-length progelatinase A to TIMP-4.** *A*, recombinant full-length gelatinase A (*lane B*, before application) (2 μg/well in 100 μl of PBS) was applied to microwell plates coated with gelatin, TIMP-2, TIMP-4, TIMP-1, or myoglobin (*Mb*) (0.5 μg/well) for 2 h as shown. After washes with PBS, 0.05% (v/v) Tween 20 to remove unbound and nonspecifically bound enzyme, bound gelatinase A was solubilized in 50 μl of 2 M urea, 2% (w/v) SDS, 0.125 M Tris-HCl, pH 6.8, and quantitated by gelatin enzymography, applying 10 μl of sample/lane. Following electrophoresis, gels were incubated for 1 h at 37 °C in 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$. *B*, serum-free conditioned culture medium from confluent ROS 17/2.8 cells (*lane B*, before application) was applied at 100 μl/well to microwell plates coated with gelatin, TIMP-2, TIMP-4, TIMP-1, or myoglobin (*Mb*) (0.5 μg/well) as shown. Unbound enzyme was removed and bound enzyme analyzed as for *A* except that digestion times were 3.3 h for *B* and 18 h for all other samples shown. *ProGL*, progelatinase A, *GL*, active gelatinase A.

but not to TIMP-1 and myoglobin (negative controls) (Fig. 4*A*). Of note, the small amount of active enzyme present (M_r = 59,000) was bound by gelatin but not by TIMP-4 or TIMP-2, indicating that the interaction observed for the proenzyme was not through the catalytic site. Proenzyme from concanavalin A treated and untreated ROS 17/2.8 cells also showed binding to TIMP-4, TIMP-2, and gelatin but no binding to TIMP-1 or myoglobin (Fig. 4*B*). However, the quantity of *recombinant* progelatinase A activity binding to microwell plates was much greater than the enzyme activity bound from ROS 17/2.8 cell conditioned medium. Although not quantitative, this was indicated by the marked difference in enzymogram digestion times required to obtain visible lysis bands of bound enzyme: 18 h for conditioned medium and 1 h for recombinant enzyme. Indeed, the majority of progelatinase A from conditioned culture medium did not bind to the microwells as shown by enzymogram analysis of the enzyme before application (digestion time of 3.3 h) (Fig. 4*B*). This indicates that most progelatinase A produced by ROS 17/2.8 cells is unavailable for binding to TIMPs, probably due to prior formation of a complex with TIMP-2.

DISCUSSION

All members of the TIMP family are characterized by their ability to inhibit MMP activity by forming essentially irreversible 1:1 molar stoichiometric complexes with the active enzymes (20, 30). However, the association of TIMPs with the proforms of the MMPs is more specific, and so far only two such interactions have been described: that of TIMP-2 with the proform of gelatinase A (22, 33–35) and that of TIMP-1 with the proform of gelatinase B (13, 45). In this report, we present evidence for the first time of a third TIMP-progelatinase interaction, that is, of the newly cloned TIMP-4 with progelatinase A.

Binding of TIMP-2 to progelatinase A has been shown to occur via the C domain of the enzyme (17, 18, 34, 36); indeed, the C domain alone can bind TIMP-2 (18, 34, 36, 37, 39). Similarly, we have shown that both progelatinase A and the rC domain alone can bind TIMP-4, indicating that the binding site in the full-length proenzyme resides mainly or entirely within the C domain. The lack of binding of the rCBD to TIMP-4 demonstrates that this domain is unlikely to be involved in complex formation. However, the contribution of N domain binding sites elsewhere in the catalytic domain cannot be precluded, because for TIMP-2, the existence of such a site or sites on the proenzyme has been shown (50). Our data suggest that the binding mechanism of TIMP-4 to the C domain of gelatinase A may be closely similar to that of TIMP-2. First, the K_d equilibrium values for the binding of rC domain to immobilized TIMP-2 and TIMP-4 on microwell plates were comparable, although binding to TIMP-2 was slightly stronger. Secondly, the elution profile of TIMP-4 following application to rC domain immobilized on an affinity column was identical to that of TIMP-2 under the same experimental conditions (39). In addition, preincubation of the rC domain with TIMP-2 (which would be expected to result in the formation of an rC domain-TIMP-2 complex) prevented subsequent binding to TIMP-4. This demonstrates that both inhibitors cannot bind simultaneously, suggesting the presence of a common or overlapping binding sites on the rC domain. Latent gelatinase A from ROS 17/2.8 cell conditioned culture medium is present mainly as an enzyme-TIMP-2 complex (22, 33). The reduced binding of this enzyme to exogenous TIMP-4 and TIMP-2 therefore further indicates competition for a common or overlapping binding sites. However, the existence of separate sites in which binding of TIMP-2 blocks TIMP-4 binding, either by steric hinderance or by inducing a conformational change in the rC domain, cannot be precluded by our data.

The apparent dissociation constant for binding of the rC domain to TIMP-2 (0.66×10^{-7} M) is somewhat higher than that previously reported for the full-length enzyme or for the binding of the C domain to cells via TIMP-2 (34, 43). This may be because additional interactions of TIMP-2 with other sites on the N domain outside of the active site and the CBD occur to increase the affinity of binding (50). Alternatively, in our experiments, TIMP-2 and TIMP-4 are bound to microwell plates, possibly via sites that are involved in binding to the rC domain, resulting in a weaker interaction. The location of the rC domain binding site on the TIMP-4 molecule requires further investigation; in the case of TIMP-2, a highly charged sequence QEFLDIEDP located at the COOH terminus of the inhibitor is proposed to occur as an exposed "tail" and to mediate binding to

progelatinase A (37). The homologous sequence in TIMP-4 (**KEF**V**DIV**Q**P**) (31) shares four conserved residues (bold type) and an additional two conservative substitutions (underlined), none of which are found in TIMP-1 or TIMP-3, suggesting that this sequence may be important in forming the binding site. The remaining nonconserved residues reduce the net negative charge of the peptide from −4 in TIMP-2 to −1 in TIMP-4, which may cause the binding of TIMP-4 to be weaker than that of TIMP-2. The location of binding sites for TIMP-2 and TIMP-4 on the rC domain are currently under investigation in our laboratory and appear to involve several positively charged clusters unique to gelatinase A on hemopexin-like modules III and IV.

The findings of this study raise the important question of the physiological role of TIMP-4 binding to progelatinase A. TIMP-4 appears to mimic TIMP-2 in this capacity, thereby implying functional redundancy between the two inhibitors. However, the expression of TIMP-4 has been shown to be highly tissue-specific (31, 32) in contrast to TIMP-2, which is widely and constitutively expressed. This implies that TIMP-4 has a unique role that is distinct to that of TIMP-2. Binding of the C domain of progelatinase to TIMP-2 is required to mediate activation of the enzyme; this is believed to occur through the formation of a trimolecular complex of MT1-MMP, TIMP-2, and proenzyme on the cell membrane (43). We speculate that binding of progelatinase A to TIMP-4 could be involved in the formation of an alternative trimolecular complex, perhaps preferentially with one of the other MT-MMPs, giving rise to an alternative activation pathway which is functional only in tissues which express TIMP-4. Formation of the MT-MMP-TIMP-2-progelatinase A trimolecular complex appears to be sequential, such that preformed TIMP-2-progelatinase A complex will not bind (43) and is therefore resistant to activation (36, 42, 43). Hence, an alternative possibility is that complex formation between progelatinase A and TIMP-4 prevents the enzyme from association with membrane bound TIMP-2 and therefore blocks activation. Interestingly, the highest level of TIMP-4 expression is in the heart (31), in which cancer metastasis rarely occurs, a process that is believed to involve the expression and activation of gelatinase A. Perhaps this is because the presence of excess TIMP-4 prevents gelatinase A activation via complex formation with the proenzyme.

In summary, we have demonstrated specific, high affinity binding of the newly described inhibitor TIMP-4 to progelatinase A. Binding appears to occur mainly via the C domain of the enzyme and to resemble that of TIMP-2. Future work will address the sites of interaction of TIMP-4 on the C-domain of gelatinase A and the physiological significance of this phenomenon.

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